Detection of somatic variants from genomic data and their role in neurodegenerative diseases

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A mi familia,

“La paciencia es la madre de la ciencia”

Refranero español

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Abstract

Somatic mutations are those that arise after the zygote is formed and are therefore inherited by a fraction of the cells of an individual. Their relevance to a handful of diseases has been known for almost half a decade and they have been extensively studied in the context of cancer, the most common disease caused by somatic mutations. Yet, their prevalence in healthy individuals, their importance in phenotypic variation or their putative role in other human disorders such as neurodegenerative diseases are still open questions. Furthermore, accurate detection of somatic variants from bulk sequencing data poses a technical challenge. This work focuses on detecting and circumventing the biases that hinder their identification in such approach. Using this knowledge, we identified somatic point mutations in the exomes of five different tissues from sporadic Parkinson disease patients. We also assessed the detection of somatic copy number variants from array CGH data using two tissues from Alzheimer disease patients. Finally, we participated in the identification of somatic variants in an extensive genomic dataset from a neurotypical individual.

Resumen

Las mutaciones somáticas son aquellas que surgen tras la formación del cigoto y son por ello heredadas por una fracción de las células de un individuo. Su importancia para algunas enfermedades se conoce desde hace casi medio siglo y se han estudiado extensamente en el contexto del cáncer, la enfermedad más común causada por mutaciones somáticas. Sin embargo, su prevalencia en individuos sanos, su importancia en la variación fenotípica, así como su potencial relevancia en otras afecciones humanas, tales como las enfermedades neurodegenerativas, son cuestiones por resolver. Asimismo, detectar variantes somáticas con precisión en datos de secuenciación de muestras homogeneizadas es complicado técnicamente. Este trabajo se centra en la detección y resolución de los sesgos que dificultan su identificación. Aplicando este conocimiento, identificamos mutaciones somáticas de una sola base en datos de secuenciación del exoma de cinco tejidos diferentes de pacientes de la enfermedad de Parkinson. También evaluamos la detección de variantes de número de copia somáticas en datos de array CGH de dos tejidos de pacientes de Alzheimer. Finalmente, hemos participado en la identificación de variantes somáticas en un amplio conjunto de datos genómicos de un individuo neurotípico.

ABBREVIATIONS

aCGH	array Comparative Genomics Hybridisation
AD	Alternative allele Depth
BAC	Bacterial Artificial Chromosome
BER	Base-Excision Repair
bp	Base pairs
BPES	Blepharophimosis-Ptosis-Epicanthus inversus Syndrome
BSMN	Brain Somatic Mosaicism Network
CADD	Combined Annotation Dependent Depletion
CGH	Comparative Genomics Hybridisation
CN	Copy Number
CNV	Copy Number Variant
COSMIC	Catalogue Of Somatic Mutations In Cancer
DNA	Deoxyribonucleic Acid
DS	Down Syndrome
DSB	Double Strand Break
EBV	Epstein-Barr virus
ENCODE	Encyclopaedia of DNA Elements
FET	Fisher Exact Test
GWAS	Genome-Wide Association Studies
HMW	High Molecular Weight
HR	Homologous Recombination
IBS	Iberian populations in Spain
IGV	Integrative Genomics Viewer
indel	Short insertion or deletion
kb	kilobase
lncRNA	long non-coding RNA
LQTS	Long-QT syndrome
MALBAC	Multiple Annealing and Looping-Based Amplification Cycles
MDA	Multiple Displacement Amplification
miRNA	micro RNA
MMR	Mismatch Repair
mRNA	messenger RNA
NAHR	Non-Allelic Homologous Recombination
ncRNA	non-coding RNA
Ne	Effective population size
NER	Nucleotide-Excision Repair
NHEJ	Non-Homologous End Joining
OMIM	Online Mendelian Inheritance in Man
PacBio	Pacific Biosciences
PCA	Principal Component Analysis
PCR	Polymerase Chain Reaction
PD	Parkinson Disease
PFC	Prefrontal Cortex
PIR	Position In Reads
piRNA	piwi-interacting RNA
RNA	Ribonucleic Acid
SD	Standard Deviation

SD	Segmental Duplication
siRNA	small interfering RNA
SMRT	Single Molecule Real Time
snoRNA	small nucleolar RNA
SNP	Single Nucleotide Polymorphism
snRNA	small nuclear RNA
SNV	Single Nucleotide Variant
STR	Short Tandem Repeat
TAD	Topologically Associating Domains
TE	Transposable Element
tRNA	transfer RNA
UV	Ultra Violet
VAF	Variant Allele Frequency
VaD	Vascular Dementia
WES	Whole Exome Sequencing
WGA	Whole Genome Amplification
WGAC	Whole Genome Assembly Comparison
WGS	Whole Genome Sequencing

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INTRODUCTION

1. The genome

The genome is defined as the complete set of genetic information of an organism. Its material substrate is the deoxyribonucleic acid (DNA), a double helix formed by two chains of nucleotides (Franklin and Gosling 1953). The strands are formed by a backbone of alternating phosphate groups and deoxyriboses, with one of the nucleobases bound to the latter (Fig. 1). There are two types of nucleobases, purines: adenine (A) and guanine (G), and pyrimidines: cytosine (C) and thymine (T). Each purine base is complementary to one pyrimidine – A with T and C with G – and linked across the strands by hydrogen bonds (Watson and Crick 1953). This way, the double helix is formed by a sequence of nucleotides on one strand and its reverse complement on the other strand. Information for multiple biological processes is encoded in the nucleotide sequence and can be precisely copied according to the base pairing rules.

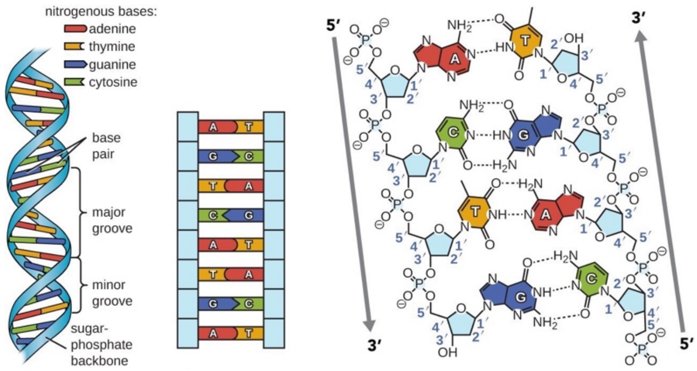


Figure 1. DNA structure. The double helix is formed by two chains of nucleotides, characterized by the nucleobase they incorporate. Backbones are closer together on one side of the helix (minor groove) than in the other (major groove). Nucleobases are linked by hydrogen bonds according to the complementary base pairing rules. The different chemical groups of the backbone create directionality from the 5′ end, with a terminal phosphate group, to the 3′ end, with a terminal hydroxyl group. (From Lumen Learning 2019)

Since guanine and cytosine are joined by three bonds and adenine and thymine are linked by just two, each pair has different properties, both *in vivo* and *in vitro*. Therefore, GC content is an important genomic feature vastly studied because it correlates with life history traits in mammals (Romiguier et al. 2010), and affects sequencing technologies (Benjamini and Speed 2012). Single nucleotide substitution changes from one purine to the other or from a pyrimidine to the other are termed transitions, whereas transversions imply a change in the nucleobase type. Even though there are more possible transversions than transitions, they are less frequent, with the transition/transversion ratio (ti/tv) of the human genome reported to be at 2.1 (Durbin et al. 2010).

1.1 Protein coding genes

Only about 1.5% of the human genome codes for proteins, with an estimated number of ~19,000 protein coding genes (Ezkurdia et al. 2014). This portion of the genome is comprised by the complete set of exons and it is called the exome.

Proteins are a fundamental type of macromolecules for living organisms. Their functions are varied, from catalyzation of chemical reactions to structural roles. They are also composed of the sequence of simpler molecules: amino acids. Only 22 amino acids make up proteins in all known organisms (Srinivasan, James, and Krzycki 2002), with 20 comprising the standard eukaryotic set. Since they have different shapes, sizes and polarities, their combination creates different 3D structures.

The information on the sequence of amino acids necessary to produce a specific protein is encoded in protein coding genes. However, in between exons – the sequence stretches that code for amino acids – eukaryotes have introns, sequences that need to be removed before being translated to proteins, a process known as splicing. Within an intron, three sites are required for splicing: the donor site at the 5′ end, the branch site near the 3′ end and the acceptor site at the 3′ end. The multiple components that form the spliceosome complex bind to these sites, which have different consensus sequences, and remove the intron, joining exons together. Splicing allows the combination of different exons from the same DNA sequence, a mechanism known as alternative splicing, which increases protein diversity.

Since many copies of the same protein need to be produced and processed at the same time, the DNA sequence is first transcribed into ribonucleic acid (RNA) molecules in the nucleus following the complementary base pairing rules (Fig. 2). RNA essentially differs from DNA in that it consists of a single strand of nucleotides and it contains uridine (U) instead of thymine. RNA has multiple key roles: when it is copied from the DNA sequence and takes the information to the ribosomes it is called messenger RNA (mRNA). Each mRNA gets spliced inside the nucleus and once processed, goes to the cytoplasm and binds to a ribosome, where translation to proteins occurs. Each triplet of nucleotides, or codon, is translated into an amino acid. A different type of RNA, the transfer RNAs (tRNAs), also participate in this process. They carry a nucleotide triplet – the anticodon – as well as one of the amino acids. When a tRNA finds the complementary codon on the mRNA, the amino acid it carries is bonded to the existing chain, synthesizing a new protein which depends on the nucleotide sequence on the processed mRNA (Fig. 2).

The correspondence between codons and amino acids is the genetic code. There are 64 possible permutations of 4 nucleotides taken in triplets (4^3) but only 20 amino acids. Although there are three stop codons, which signal for translation stop (Fig. 3), 61 codons code for amino acids. For this reason, the genetic code is redundant, i.e., multiple codons translate to the same amino acid. However, this redundancy is not arbitrary, changes in the third nucleotide of a codon frequently encode for the same amino acid. This codon degeneracy is mediated by tRNA chemical modifications that allow certain nucleotides to wobble, that is, to pair with multiple nucleotides (Agris, Vendeix, and Graham 2007).

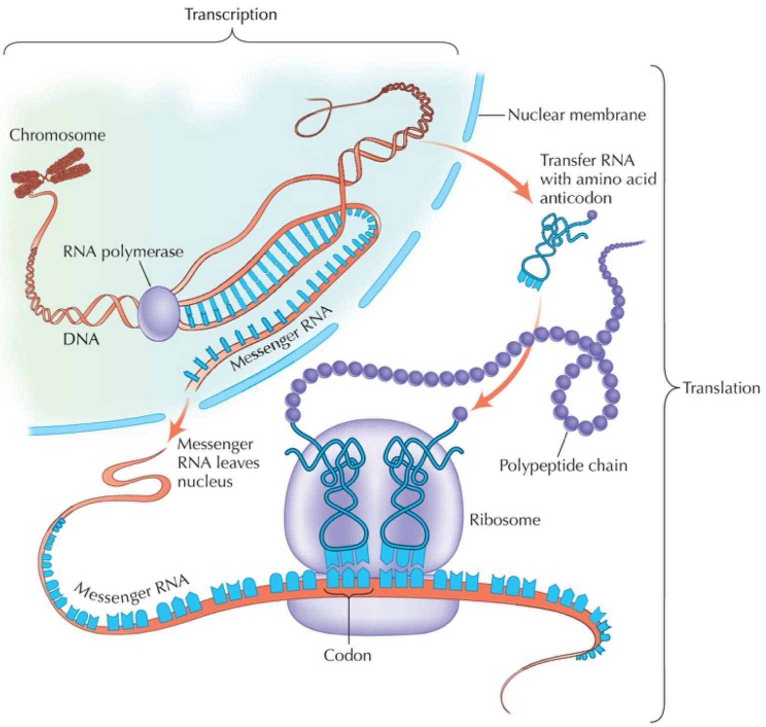
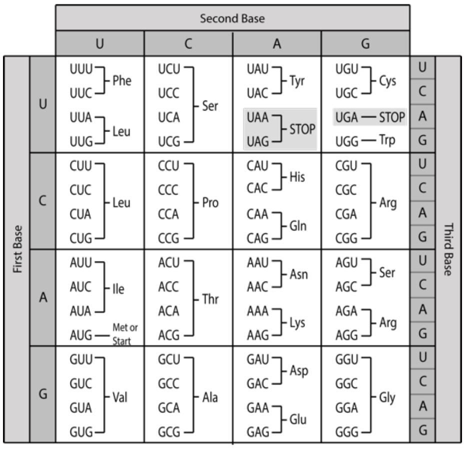


Figure 2. Transcription and translation. DNA is transcribed to messenger RNA (mRNA) inside the nucleus. Transfer RNAs (tRNAs) are also transcribed from genomic DNA. For translation to proteins, mRNA binds to a ribosome, where each codon is matched to an anticodon from a tRNA and the amino acid it carries is incorporated to the polypeptide chain. (From Barton 2007)



First Base		Second Base U	Second Base C	Second Base A	Second Base G	Third Base
U		UUU Phe	UCU Ser	UAU Tyr	UGU Cys	U
		UUC Phe	UCC Ser	UAC Tyr	UGC Cys	C
		UUA Leu	UCA Ser	UAA STOP	UGA — STOP	A
		UUG Leu	UCG Ser	UAG STOP	UGG — Trp	G
C		CUU Leu	CCU Pro	CAU His	CGU Arg	U
		CUC Leu	CCC Pro	CAC His	CGC Arg	C
		CUA Leu	CCA Pro	CAA Gln	CGA Arg	A
		CUG Leu	CCG Pro	CAG Gln	CGG Arg	G
A		AUU Ile	ACU Thr	AAU Asn	AGU Ser	U
		AUC Ile	ACC Thr	AAC Asn	AGC Ser	C
		AUA Ile	ACA Thr	AAA Lys	AGA Arg	A
		AUG — Met or Start	ACG Thr	AAG Lys	AGG Arg	G
G		GUU Val	GCU Ala	GAU Asp	GGU Gly	U
		GUC Val	GCC Ala	GAC Asp	GGC Gly	C
		GUA Val	GCA Ala	GAA Glu	GGA Gly	A
		GUG Val	GCG Ala	GAG Glu	GGG Gly	G

Figure 3. The genetic code. Each triplet of nucleotides in the mRNA, or RNA codon, codifies for an amino acid. AUG is the starting codon, which always codes for methionine, and there are three different stop codons. (By Sarah Greenwood)

This is why certain point mutations, mostly in the third codon position, are termed *synonymous mutations*: their replacement does not result in a different amino acid being incorporated, maintaining the resulting protein unchanged. On the other hand, *nonsynonymous mutations* occur when nucleotide substitutions change the produced protein. They are known as *missense mutations* when they change the amino acid sequence, *nonsense mutations* when they create a new stop codon or *readthrough mutations* when they remove a stop codon, producing a longer protein. Also, point mutations in the introns can modify the consensus sites, altering splicing, which can result in aberrant proteins.

Besides single nucleotide variants (SNVs), small insertions or deletions (indels) in the exons can also change the resulting protein. Often, the number of base pairs added or removed is not a multiple of 3, changing the grouping of the following nucleotides in codons. This is why they are called frame-shift mutations.

1.2 Non-coding DNA

The remaining more than 3 billion base pairs of the genome are termed non-coding DNA. It has been known for many decades that only a small fraction of the genome is protein-coding, which was later confirmed by the Human Genome Project (International Human Genome Sequencing Consortium 2001). Besides promoters – the regions adjacent to protein-coding genes where the enzymes that catalyze replication or transcription attach – it was difficult to assign a function to the rest of the genome, which is mostly formed by repetitive elements. Moreover, the huge variability in genome size, even between closely related species, points towards much of the genome not having a function (Palazzo and Gregory 2014).

Still, a structural function was proposed early on. In fact exactly at the same time that the term “junk DNA” was coined (Ohno 1972). The original idea was that placing genes far away from the centromeres allows for duplications or deletions of centromeric regions along evolution without damaging consequences, which result in the chromosomal rearrangements we oftentimes observe accompanying speciation. Also, the existence of non-coding chromatin in between protein-coding genes ensures that the consequences of nonsense or frame-shift mutations are contained to one single locus.

More recently, chromosome conformation capture methods such as Hi-C (Dekker et al. 2002; Lieberman-Aiden et al. 2009) have encouraged the scientific community to explore the genome’s 3D nuclear architecture and how it relates to function. It has been shown that during the interphase, chromosomes reside in specific spaces, called chromosome territories (reviewed in Cremer and Cremer 2001). In a smaller scale, the genome organizes in domains with increased frequency of internal interactions (Fig. 4), which are termed topologically associating domains (TADs) (Dixon et al. 2012; Nora et al. 2012; Sexton et al. 2012). They are delimited by CTCF binding motifs, regions that allow the attachment of the homonymous insulator protein, which through a process that is not yet fully unraveled, creates chromatin loops (Rao et al. 2014). TADs bring together gene promoters and enhancers (Shen et al. 2012) and share chromatin features such as coordinated gene expression or replication timing (Dixon et al. 2012). This is why their disruption can cause disease (Lupiáñez, Spielmann, and Mundlos 2016), hinting at how little we know about the role of most genomic regions and making it difficult to judge the existence of non-coding DNA functions or lack thereof.

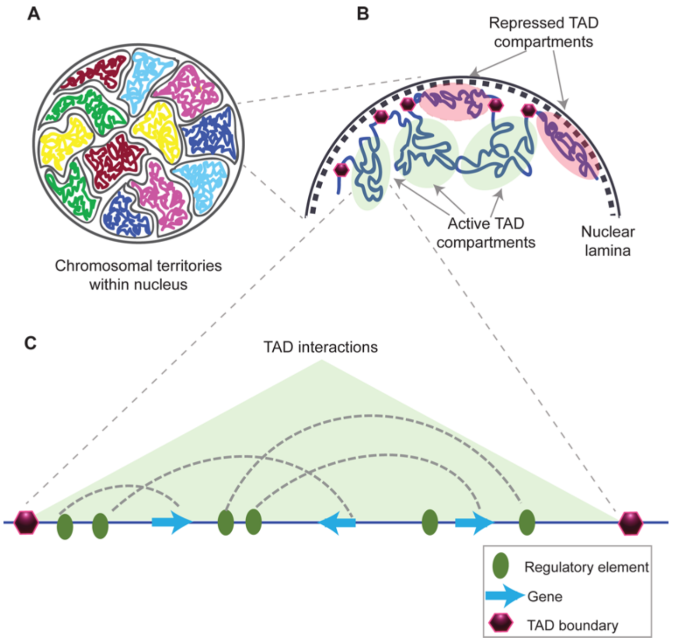


Figure 4. Structural organization of chromatin. A. In the interphase, chromosomes occupy specific nuclear spaces, termed chromosomal territories. **B.** Chromosomes are subdivided into topological associated domains (TAD). TADs with repressed transcriptional activity tend to be associated with the nuclear lamina (dashed inner line), while active TADs tend to be in the nuclear interior. **C.** Each TAD is flanked by CTCF binding motifs called TAD boundaries (purple hexagon). (From Matharu and Ahituv 2015)

The most prominent component of the human genome, and more so of larger genomes, are transposable elements (TEs). These DNA sequences are able to copy and insert themselves into new genomic regions (McClintock 1950). They can do this because they encode transposase, the enzyme that catalyzes these reactions. Since TEs have control over their own transmission, they have been labelled, together with regions of similar characteristics, as *selfish genomic elements* (Doolittle and Sapienza 1980; Orgel and Crick 1980; Ågren and Clark 2018). This implies that the reason they are so frequent in genomes is because they self-copy, so to some extent, they expand independently of their effect on fitness. Nonetheless, because TEs insert frequently in the genome, occasionally they become functional, fine-tuning the transcriptome (Cowley and Oakey 2013) or even influencing local adaptation by altering splicing (González et al. 2010). Unsurprisingly, the same alterations of splicing can derive in disease (Hancks and Kazazian 2016). Moreover, they can create novel transcription binding sites, such as CTCF sites, which can alter genome function and architecture (Bourque et al. 2008; Merkenschlager and Odom 2013).

However, there are specific non-coding sequences whose role we do understand. An important group are RNA molecules, the genomic regions that are transcribed to various forms of RNA and carry out their function without being translated to proteins. Besides mRNA, tRNA and ribosomal RNA (rRNA), the RNAs that make translation possible, a myriad of non-coding RNAs (ncRNAs) have essential regulatory roles. Long non-coding RNAs (lncRNAs) appear to be involved in transcription regulation by recruiting transcription factors (Feng et al. 2006) and tethering RNA binding proteins (Wang et al. 2008). The most famous lncRNA is the X inactivate-specific transcript (XIST), which inactivates one chromosome X in females for dosage compensation (Rastan 1994). MicroRNAs (miRNAs) are involved in mRNA silencing (Fig. 5). Because double-stranded RNA molecules are degraded, miRNAs are complementary to the target mRNA so that their base-pairing induces specific mRNA cleavage (Lau et al. 2001). Similarly, small interfering RNAs (siRNAs) are double stranded molecules that also induce complementary mRNA silencing (Hamilton and Baulcombe 1999).

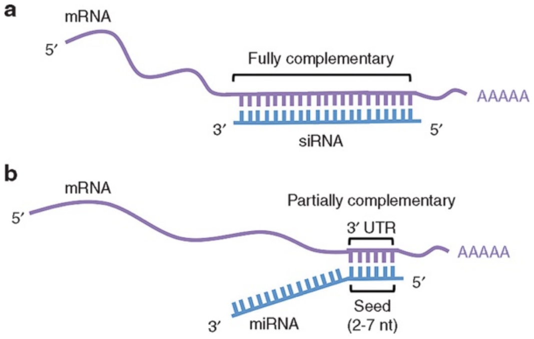


Figure 5. Target recognition by siRNA and miRNA. **A.** siRNA is usually fully complementary to the coding region of its target mRNA. **B.** miRNA is partially complementary to its target miRNA. Complementary binding usually occurs at the seed region of miRNA and the 3' UTR of the target mRNA. (From Lam et al. 2015)

Small nuclear RNAs (snRNAs) are instead part of the spliceosome, a complex that processes the pre-mRNA in the nucleus (Will and Lührmann 2011). A subset of snRNAs, small nucleolar RNAs (snoRNAs) are located in the nucleolus, where they guide chemical modifications of other RNAs (Samarsky et al. 1998). Finally, piwi-interacting RNAs (piRNAs) are the largest group of small ncRNA. Their main function is to protect the integrity of the genome by restricting the mobilization of TEs (Siomi et al. 2011) and many have interesting names, such as the *flamenco* locus, which determines whether the transposable element *gypsy* “dances” (Prud’homme et al. 1995).

Further, introns are also involved in the regulation of gene expression. They do so via multiple mechanisms, including altering transcription timing (Swinburne and

Silver 2008) or promoting the export of mRNAs to the cytoplasm (Valencia, Dias, and Reed 2008). They are also responsible for mRNA quality control (Lee et al. 2009). Because some of these intronic functions depend on intron length rather than on sequence (Chorev et al. 2017), the detection of functional introns is complex, making it challenging to estimate the proportion of the genome carrying out these functions.

A different type of repetitive sequences, short tandem repeats (STRs) consist of a simple DNA motif – usually from two to thirteen base pairs long – repeated a variable number of times. When the motif is just one nucleotide, and therefore the STR contains the same nucleotide repeated multiple times, it is termed a homopolymer. Whether homopolymers are STRs or not is a matter of debate. Since STRs are repetitive, during replication, the different repetitions can pair between them, making DNA polymerase replicate the region over again, a process known as replication slippage (Kornberg et al. 1964). Most of the time, these errors are repaired by nucleotide excision repair (see 2.3) (Strand et al. 1993), but still, together with other mutational mechanisms (Fan and Chu 2007), this makes them highly mutable genomic regions. Precisely for this reason, their sequencing is widespread in forensic analysis (Tautz 1989); they are so variable within populations that the characterization of 13 to 17 known loci is used as a molecular fingerprint.

STRs have been shown to be involved in gene expression regulation (Gymrek et al. 2012), in altering recombination frequency (Wahls, Wallace, and Moore 1990) and in generation of nucleosome positioning signals (Wang and Griffith 1995). Furthermore, the expansion of certain trinucleotides within genes causes multiple disorders. Perhaps the most famous of them is Huntington disease, in which the number of CAG repeats in the *HTT* gene (MacDonald et al. 1993) determines its stability in replication. The disease is developed when the number of repeats surpasses 40 and more copies increase its severity (Aziz et al. 2009). Other trinucleotide repeat disorders include Fragile X syndrome, myotonic dystrophy or spinocerebellar ataxia (Orr and Zoghbi 2007).

Besides functions determined by DNA sequence, the role of multiple genomic regions is indicated by their epigenetic marks. Certain chemical modifications to the DNA and histone proteins – those that package DNA around them, forming nucleosomes – determine how accessible chromatin is, regulating its level of transcription (Fig. 6). This is one of the mechanisms by which the same DNA sequence in different cell types can result in different transcriptomes. The

Encyclopedia of DNA Elements (ENCODE) project (Birney et al. 2007) and the Roadmap Epigenomics Mapping Consortium (Bernstein et al. 2010) have worked towards identifying functional elements in the human genome. Besides already known promoter and enhancer regions, they discovered new candidate regulatory elements and assigned different states to chromatin depending on the combination of its epigenetic marks. This knowledge allowed for the interpretation of non-coding variants previously linked to disease (Maurano et al. 2012; Ward and Kellis 2012).

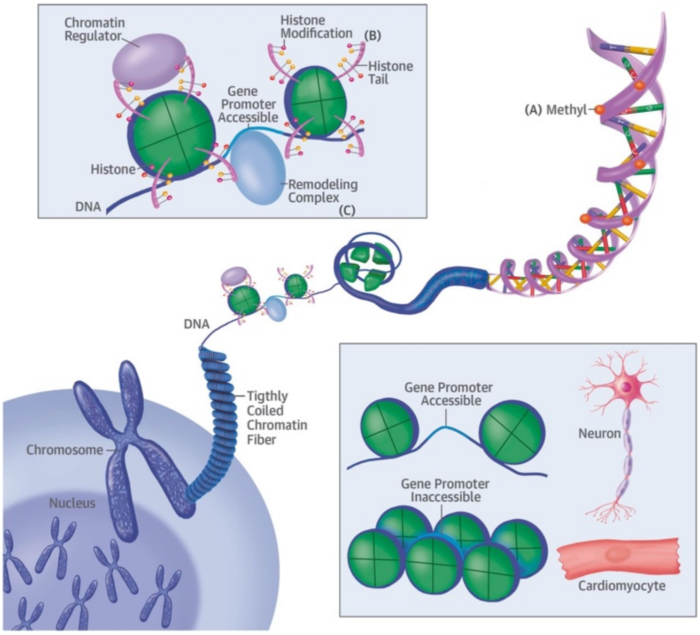


Figure 6. Epigenetic modifications. Epigenetic marks include DNA methylation (A) and histone modifications (B) such as methylation or acetylation of some histone amino acids. They determine how compacted chromatin is, which makes it more or less accessible to cell machinery, regulating expression. (From van der Harst, de Windt, and Chambers 2017)

2. DNA repair

Cells reproduce by division, the process by which a parent cell gives rise to two daughter cells. Since there is one copy of the genome in each cell, DNA needs to be replicated so that each daughter cell has its own copy. Multiple enzymes are required for replication, from those that recognize replication origins to many directly involved in DNA synthesis. After DNA primases and helicases separate both DNA strands, DNA polymerases catalyze the polymerization of a new DNA strand using one of the existing strands as a template. This way, each daughter cell inherits a double strand composed by one of the original strands and a newly synthesized one.

2.1. Replication errors

Considering the genome is large and there are many cells in an organism – $3 \cdot 10^{13}$ in an adult human body (Sender, Fuchs, and Milo 2016) – any error rate when copying DNA, even if low, will produce many mutation. Since there is only one copy of the nuclear genome in each cell, changes in its sequence can be of great importance. Thus, cells have suffered a big selective pressure to evolve mechanisms that help avoid and correct errors. The polymerases most commonly used by eukaryotes have high fidelity, with replication error rates of ~10^{-5} for Polδ and from 10^{-3} to 10^{-4} for Polα and Polβ (D. C. Thomas et al. 1991; Osheroff et al. 1999). Because many subsequent repair mechanisms ensure the correction of replication errors and spontaneous or environmental DNA damage, human germline mutation rate is much lower, roughly 10^{-9} (Michael Lynch 2010).

During replication itself, DNA polymerases can correct misincorporated bases. This process, known as proofreading, is a type of excision repair (see 2.3) in which polymerases use their 3′→5′ exonuclease activity to remove a mismatched nucleotide. All three bacterial DNA polymerases have this ability, whereas in eukaryotes only those enzymes involved in elongation have it. The vast majority of replication errors are recognized by proofreading, so that after a mistake, DNA polymerases reverse their direction to excise the mismatched base. Following base excision, polymerases re-insert the correct nucleotide. The few cases that escape this repair mechanism, are then recognized by the mismatch repair system (see 2.3).

2.2. DNA damage

DNA damage can be spontaneous or induced by environmental factors. The most common type of spontaneous damage is deamination of 5-methylcytosine (methylated cytosine) (Shen, Rideout, and Jones 1994), which results in thymine and ammonia, producing a C>T substitution. Unmethylated cytosines can suffer deamination too, resulting in uracil bases, which would also give rise to a C>T substitution. Although the deamination of purines is very infrequent in comparison (Tomas Lindahl 1993), guanine deamination results in xanthine, which base-pairs with thymine, producing a G>A substitution, and adenine deamination produces a hypoxanthine which base-pairs with cytosine, resulting in an A>G substitution.

Another type of spontaneous damage is depurination, or the loss of the nucleobase at purine sites, adenine and guanine, by the cleavage of the β-N-glycosidic bond, which is especially susceptible to hydrolysis (Lindahl and Nyberg 1972), creating an apurinic site that decreases fidelity of DNA replication (Shearman and Loeb 1977).

Ultraviolet (UV) radiation is one of the main sources of induced damage. UV light induces the appearance of covalent bonds between consecutive pyrimidine nucleotides, cytosine and thymine, producing dimers (Setlow and Carrier 1966), which are mutagenic if left unrepaired and the main cause of human melanomas (Nelson and Nelson 1957; Holman et al. 1986; Østerlind et al. 1988).

Alkylating agents, such as mustard gas, can transfer methyl or ethyl groups to a DNA base (Lawley and Brookes 1967). When guanines are alkylated, they form complementary base pairs with thymine, creating a G>A substitution.

Oxidation affects most commonly guanines, because they have a lower reduction potential (Steen Steenken and Jovanovic 1997). Oxidized guanines abnormally pair with adenine, producing a G>T substitution (Shibutani, Takeshita, and Grollman 1991).

Further, double strand breaks (DSBs) occur frequently after exposure to ionizing radiation, induced by certain chemical agents, due to cross-overs during replication (Haber 1999; Karran 2000) or as a normal step of recombination in meiosis. DSBs are especially dangerous for cells because they can result in big duplications or deletions, as well as chromosomal rearrangements and even cell death (Carson et al. 1986).

2.3. Repair systems

Besides DNA polymerases proofreading, any remaining error or damage is corrected by a series of mechanisms, depending on the type of alteration.

Direct reversal of damage

Occasionally, damage is directly reversed. For example, methylation of guanines is reversed by the protein methyl guanine methyl transferase (MGMT) (Yarosh et al. 1984), crucial for genome stability. Also, many organisms use light energy for photoreactivation, a process by which photolyase directly reverses pyrimidine dimers (Sancar 1994). This enzyme is absent in placental mammals, including humans (Kato et al. 1994), and other small effective population size (N_e) eukaryotes (Lucas-Lledó and Lynch 2009) which use nucleotide excision repair to resolve the dimers instead.

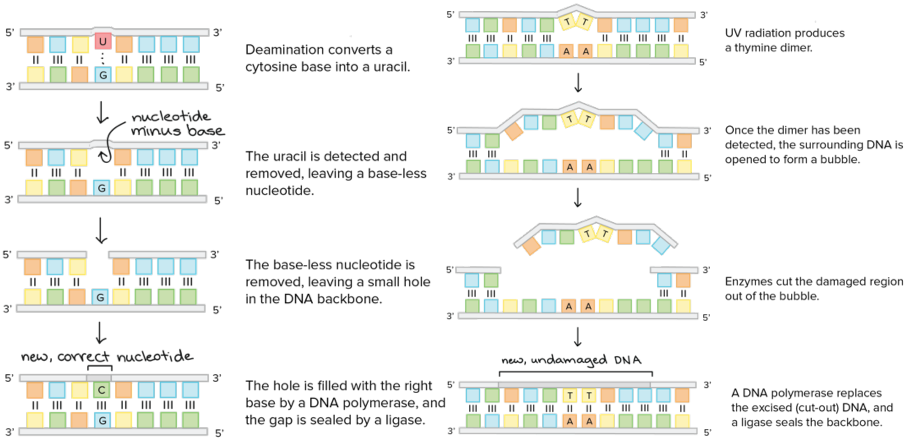
Single strand damage

Excision repair occurs when errors are removed, and the sequence is resynthesized according to the correct strand. We classify these mechanisms in three main types:

Base-excision repair (BER) (Fig. 7, left) is used when just the base itself is incorrect and it is obvious. Hence, presence of uracil bases (from cytosine deamination) in the DNA, oxidized guanines, alkylated or deaminated bases are all corrected through this system. The incorrect base is recognized and removed from the deoxyribose by DNA glycosylases (Tomas Lindahl 1982) and then the remaining deoxyribose is removed so that DNA polymerase and ligase can fill and close the gap, respectively (Seeberg, Eide, and Bjørås 1995).

Nucleotide-excision repair (NER) (Fig. 7, right panel) recognizes damaged regions because of the changes they produce to the DNA structure. This is the way T-T dimers resulting from UV damage are corrected in humans and other placental mammals. Nucleases and helicases remove an oligonucleotide including the damaged region and again DNA polymerase and ligase fill and close the gap (de Laat, Jaspers, and Hoeijmakers 1999).

Mismatch repair (MMR) is used when there is just a mismatch between bases. In this case the incorrect DNA sequence is not as apparent, but the original strand must be recognized in order to remove the erroneous base. These are the errors that escape from DNA polymerase proofreading. Because they happen during or right after replication, single-strand breaks that are only present in the newly synthesized strand are used as a mark (Kolodner and Marsischky 1999) to guide the process in mammalian cells.



5'
3'
U
G
Deamination converts a cytosine base into a uracil.
nucleotide minus base
The uracil is detected and removed, leaving a base-less nucleotide.
The base-less nucleotide is removed, leaving a small hole in the DNA backbone.
new, correct nucleotide
C
The hole is filled with the right base by a DNA polymerase, and the gap is sealed by a ligase.
T
A
UV radiation produces a thymine dimer.
Once the dimer has been detected, the surrounding DNA is opened to form a bubble.
Enzymes cut the damaged region out of the bubble.
new, undamaged DNA
A DNA polymerase replaces the excised (cut-out) DNA, and a ligase seals the backbone.

Figure 7. Base and nucleotide excision repair. Base excision repair (left) and nucleotide excision repair (right). (From Khan Academy 2019)

Double strand breaks

Double strand breaks (DSBs) are repaired by two different mechanisms: non-homologous end joining (NHEJ) and homology directed repair.

In NHEJ, DNA ligase IV, together with multiple other proteins, directly joins the ends of the broken DNA strands without the need for extensive homology between them. This process is heavily influenced by the stage of cell cycle (Moore and Haber 1996) and although it can be somewhat accurate, it is, in general, a mutagenic process, which can lead to translocations or deletions (Hiom 1999).

On the other hand, homology directed repair, or homologous recombination (HR), occurs when a homologous sequence is used as a template for repairing the break, resulting in higher accuracy repairs. A protein fundamental for HR, RAD51, searches the genome for an intact copy of the broken DNA that is used to retrieve the lost information (Houtgraaf, Versmissen, and van der Giessen 2006). Ideally, the template is the sister chromatid, so the sequence is repaired accurately. If the two sequences are not exactly homologous, it can result in gene conversion. Allelic gene conversion occurs when a strand carrying the other allele is used as a template, overwriting the original allele. However, if the repair is guided by a paralogous sequence, non-allelic homologous recombination (NAHR) occurs. Low-copy repeats or segmental duplications (SDs) – sequences 10-400 kb long with 95-97% identity – are the hotspots for NAHR, predisposing those regions to copy number variation and chromosomal rearrangements (Stankiewicz and Lupski 2002) (Fig. 8). NAHR accounts for most of the recurrent rearrangements: those that share a similar size, show clustering of breakpoints, and recur in multiple individuals (Gu, Zhang, and Lupski 2008).

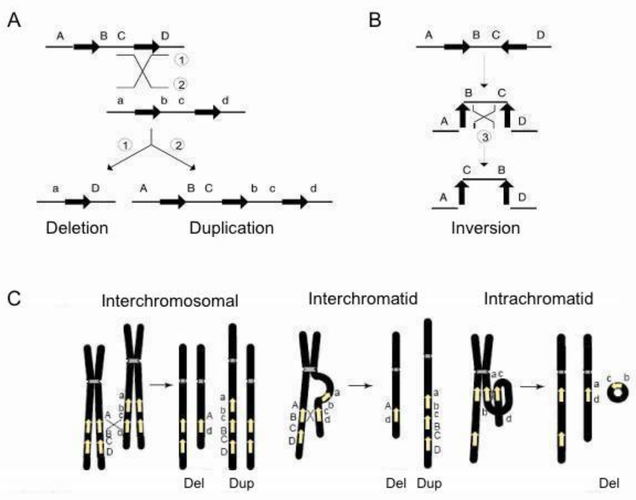


Figure 8. Genomic rearrangements resulting from NAHR between segmental duplications. Segmental duplications are depicted as arrows and the different loci are represented by letters. **A.** Recombination between direct repeats can result in deletion and duplication. **B.** Recombination between inverted repeats results in inversions. **C.** Types of NAHR depending on the involved sequences location and their consequences. (Modified from Gu, Zhang, and Lupski 2008)

Besides its relevance for resolving recombination, DSB repair is crucial for restoring collapsed replication forks (Saleh-Gohari et al. 2005). Its importance is evidenced by the fact that *BRCA1*, a gene whose mutations result in increased risk of breast cancer, is involved in multiple of these mechanisms (J. Zhang and Powell 2005).

Further, neuronal activity causes the formation of DSBs within the promoters of early-response genes, those that are rapidly activated in response to a wide variety of stimuli (Madabhushi et al. 2015b). These genes already display the hallmarks of active transcription, such as RNA polymerase II at the transcription start site, before stimulation. With neuronal activity, histone methylation and transcription factor binding are minimally altered (T.-K. Kim et al. 2010). DSBs allow the interaction of promoters with early-response genes, producing their expression. This shows how fundamental DSB repair is in the central nervous system.

Nonetheless, all the above-mentioned repair mechanisms are not infallible, in fact, error rates can be reduced only as long as they provide a fitness advantage greater than the power of genetic drift, which for species with small N_e is high. This implies that the lower bound on the mutation rate is not set by physiological or biochemical limitations, but by the inability of selection to push it lower (Michael Lynch 2010). All these errors accumulate during embryonic development and even during adult tissue proliferation and maintenance. They are shared by all the descendants of the cell where they appeared, including germ cells when they belong to the mutant lineage.

3. Early embryonic development

Sexual organisms generate gametes through meiosis, a process that separates homologous chromosomes to produce cells with half the ploidy, such that the fusion of two of these sexual cells at fertilization results in a single cell, the zygote, which will divide and develop to produce a new individual, in a process known as embryogenesis. Especially relevant to this work is human embryonic development.

3.1. Embryonic development stages

Cleavage

After fertilization, the zygote is confined inside the zona pellucida, the glycoprotein layer that surrounded the oocyte, which limits its growth in size and avoids premature implantation (W. Liu et al. 2017). In mammals, the zygote starts to divide at a pace of approximately a division per day during the first two days, a slower rate than other metazoans (O'Farrell, Stumpff, and Tin Su 2004). At this stage, cells do not grow between divisions, hence the term cleavage. After the 2-cell stage (Fig. 9, left), mammalian cleavage is asynchronous, meaning that one of the cells divides first, forming a 3-cell embryo (Kelly, Mulnard, and Graham 1978). By this stage, cells are called blastomeres (from ancient Greek *blastos*, germ or sprout) and they stay aggregated into an undifferentiated sphere (Fig. 9, middle). At the 8-cell stage, embryos enter compaction, a phase when blastomeres join with gap and tight cell junctions (Ducibella and Anderson 1975). When the embryo has approximately 12 to 32 cells, it is called a morula because of its resemblance to a mulberry.

Blastulation

Then, a cavity in the middle of the morula starts to form, the blastocoel. It is produced by the pumping of sodium into the middle of the sphere, which pulls in water osmotically (Manejwala, Cragoe, and Schultz 1989). The accumulated liquid makes the zygote grow, helping it hatch the zona pellucida. At this moment the embryo is referred to as a blastocyst. Then, cells start to differentiate between those in the outer layer, the trophoblast, and those grouped on the inside contacting the trophoblast, the inner cell mass (Fig. 9, right). The region where the inner cell mass is attached to is the embryonic pole. The contribution of each blastomere from the 4-cell stage to the inner cell mass and the trophoblast is not clear (Zernicka-Goetz 2006), but the general consensus is that some positional bias exists in guiding this commitment (Zernicka-Goetz, Morris, and Bruce 2009). The inner cell mass cells will give rise to the body of the embryo itself as well as some extraembryonic structures, such as the umbilical cord. On the other hand, trophoblast cells exclusively form extraembryonic tissues, like the outer layer of the placenta.

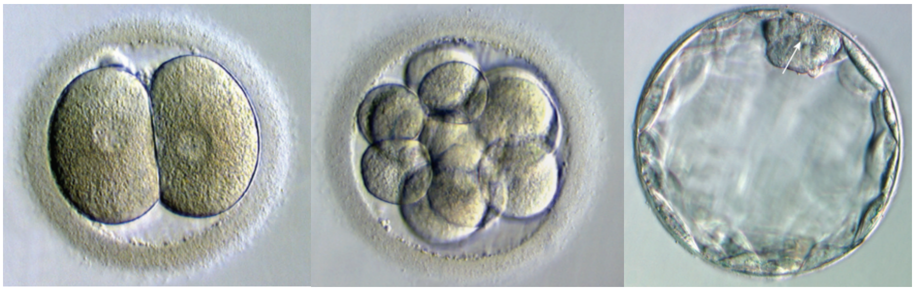


Figure 9. Photomicrographs of human embryos. Left: Two blastomeres are visible inside the zona pellucida. **Middle:** Morula with 12 visible cells. **Right:** Blastocyst with the trophoblast cells on the periphery and the inner cell mass marked by the arrow. (From Veeck and Zaninovic 2003)

Implantation

Approximately 6 days after fertilization, implantation into the uterine wall starts. Because mammalian embryos depend on maternal sustenance, after hatching the zona pellucida, the expanded blastocyst attaches to the endometrial epithelium at the embryonic pole. After the first contact, or apposition, the trophoblast cells that are near the inner cell mass fuse to form the syncytiotrophoblast, maintaining a layer of proliferative cells underneath, which also derive from the trophoblast, called the cytotrophoblast (Fig. 10A) (Enders and Schlafke 1969). The syncytiotrophoblast fusion is assisted by syncytin, a protein whose gene was inserted into the genome of an ancestor of all catarrhines by a retrovirus. Along evolution, other mammals have also exapted similar retroviral sequences for placentation (Lavialle et al. 2013).

Projections of the syncytiotrophoblast, called villi, insert between the uterine epithelial cells, and after penetrating the basal lamina, they eventually make their way into the endometrial stroma, the connective tissue beneath the epithelium. This highly invasive tissue erodes into the blood vessels of the uterus, making maternal blood fill small spaces previously formed in the syncytiotrophoblast called lacunae (Cross, Werb, and Fisher 1994). This is when some blood can leak, producing implantation spotting. Then, the decidual reaction occurs: maternal connective tissue cells swell up due to the accumulation of glycogen and lipid droplets that will be transferred to the embryo (Wislocki and Dempsey 1948). This, together with maternal arterial changes and both maternal and embryonic hormonal release, inhibit an overly aggressive invasion (Kliman 2000) and create an immunologically privileged site for the embryo (Xu et al. 2017). Later on, the maternal epithelium heals, enclosing the embryo in the stroma (Fig. 10B).

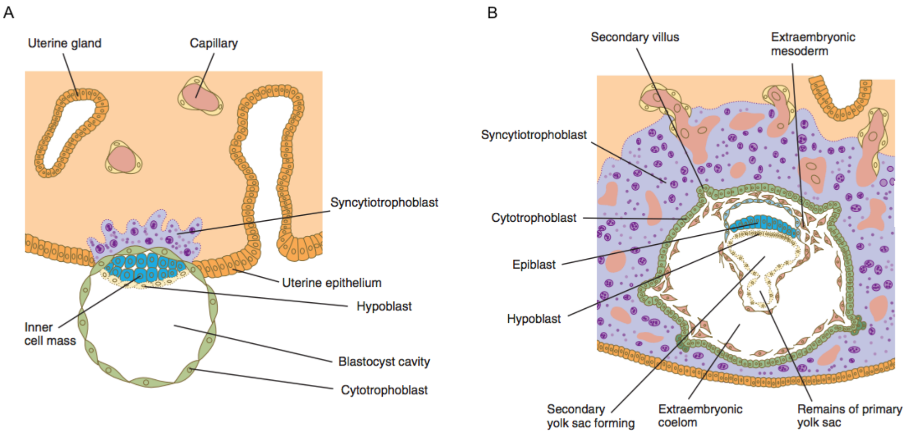


Figure 10. Embryo implantation. A. At 5-6 days post fertilization, implantation starts. Maternal tissues are depicted in orange. The syncytiotrophoblast is starting to appear and invade the endometrium. At the same time, the hypoblast and epiblast start to differentiate. **B.** At 11-12 days post fertilization, implantation is complete. Maternal capillaries have been eroded into, uterine epithelium has closed, and, in the embryo, the amniotic cavity has opened (blue bubble) and the hypoblast has formed the primary yolk sac. (From Carlson 2014)

Formation of the embryonic disk

At the same time that the syncytiotrophoblast starts to form, some inner cell mass cells form a ventral layer, constituting the hypoblast, or primitive endoderm. The upper part of the inner cell mass is known as epiblast and also forms an epithelial-like sheet (Fig. 12A). Whether an inner cell mass cell forms part of one or other layer is determined by the expression of two transcription factors, NANOG and GATA6. These two factors are initially expressed in an overlapping manner. The earliest stages of cell differentiation seem to be dominated by stochastic fluctuations of these transcription factors producing what is known as the salt-and-pepper stage (Fig. 11A). Then, through cell sorting, NANOG expressing cells form the epiblast whereas GATA6 expressing cells commit to the hypoblast by the regulation of the expression levels of fibroblast growth factor 4 (FGF4) and FGF receptor (Schrode et al. 2014). This way, a bilaminar disk is formed. Later on, a layer from the epiblast, called the amnion, separates from it, leaving the amniotic cavity in between. At the same time, cells from the hypoblast begin to spread and line the cytotrophoblast from the inside, forming the parietal endoderm, which once closed is called the yolk sac, the first site where hematopoiesis occurs (Fig. 11B) (Palis and Yoder 2001).

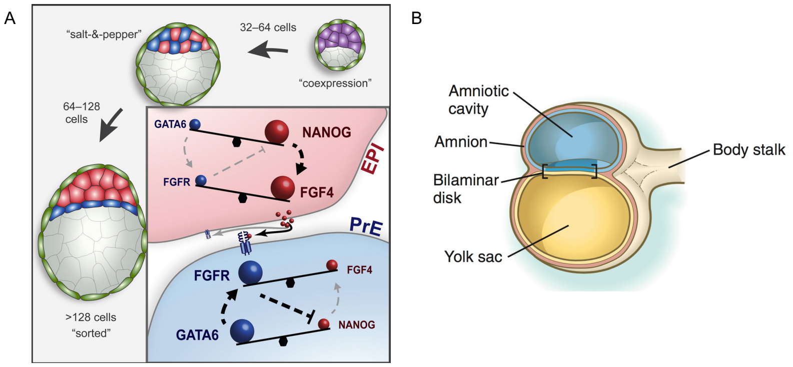


Figure 11. Development of the bilaminar disk. A. The transcription factors NANOG and GATA6 determine inner cell mass differentiation into epiblast cells (red, EPI) and hypoblast cells (blue, PrE) through the regulation of FGF. **B.** The amniotic cavity is formed by the cavitation of epiblast cells (blue) whereas the yolk sac derives from migrating hypoblast cells (yellow). The body stalk derives from the extraembryonic mesoderm and will give rise to the umbilical cord. (From Schrode et al. 2014 and Carlson 2014, respectively)

Gastrulation

This process starts with the formation of the primitive streak (Fig. 12C), a structure resulting from the loss of basal lamina and epithelial to mesenchymal transition of epiblast cells (Williams et al. 2012). At this stage, cell cycles are very rapid, as short as 2.2 hours (Snow 1977) and cells start to migrate. Cells ingress into the streak while the epiblast epithelial sheet is maintained. The first cells leaving the posterior part of the streak give rise to the extraembryonic mesoderm, which lies between the trophoblast and yolk sac and forms the body stalk (Fig. 11B), which later will become the umbilical cord, as well as give rise to the germ cells. A more anterior wave of mesoderm forms the paraxial, lateral plate, and cardiac mesoderm; structures that will give rise to the mesodermal tissues of the embryo itself. A final, anteriormost wave gives rise to the notochord (the mesodermal structure that together with ectoderm will form neural tissues) as well as to the embryonic endoderm, which will form the gut. Cells remaining in the epiblast constitute the embryonic ectoderm (Fig. 12D and E).

However, little is known about the movements cells undergo to create these layers and which are the mechanisms responsible. A study on chick embryo gastrulation showed that cells destined to different structures follow defined pathways of movement, which appear to correlate more closely with the tissue to which they would contribute than to their position in the streak at the time of labelling (Psychoyos and Stern 1996).

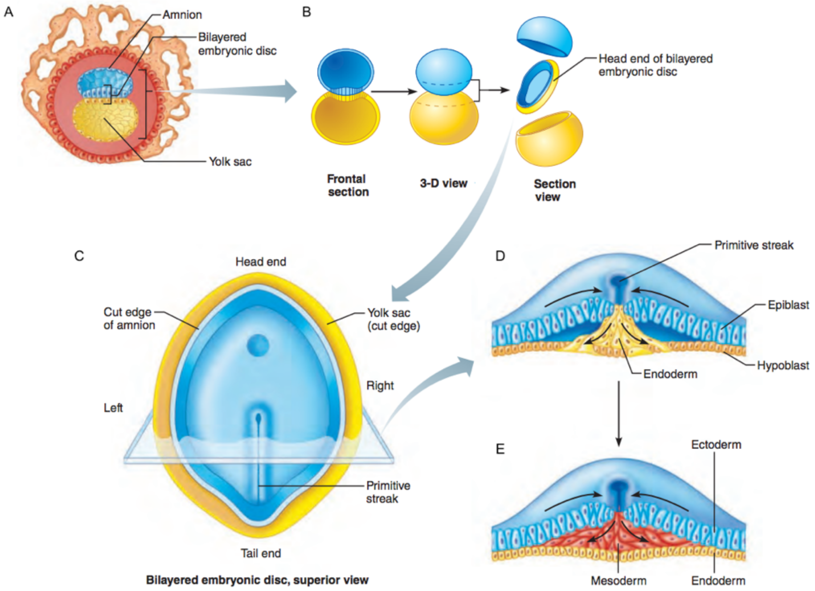


Figure 12. Gastrulation. A. Frontal section of the implanted embryo. The syncytiotrophoblast (in orange) and the cytotrophoblast (in red) surrounding the bilaminar disk. B. Orientating diagrams, showcasing the opposition of the hypoblast (yellow) and the epiblast (blue). C. Formation of the primitive streak in the bilaminar disk. D. Cells migrating along the primitive streak first form the endoderm, which mixes with the hypoblast and later (in E) the mesoderm. (From Marieb and Hoehn 2013)

Organogenesis

The three germ layers differentiate at gastrulation: ectoderm, mesoderm and endoderm. Afterwards, complex processes and rearrangements need to take place in order to produce all the body organs and organ systems (Fig. 13). Briefly, ectoderm will give rise to the outermost layer of skin, central and peripheral nervous systems, eyes, inner ear, and several connective tissues. Mesoderm will give rise to the circulatory system, including the heart and spleen, cartilage, bones, skeletal muscle, dermis, kidneys and gonads. Endoderm will give rise to the epithelial lining of the gastrointestinal track as well as the respiratory tract, the thyroid, thymus, pancreas and bladder.

Interestingly, germ cell determination occurs somewhat far from the embryo proper. Primordial germ cells derive from the extraembryonic mesoderm and commit to their lineage at the allantois, an evagination of the body stalk (Chiquoine 1954). Once specified, they first migrate to the hindgut and finally to the gonads (Tilgner et al. 2008). On their way, they proliferate at a moderate pace, each 16 hours (Tam and Snow 1981), and undergo extensive reprogramming of the epigenome, such as the removal of gene imprinting (Tilgner et al. 2008).

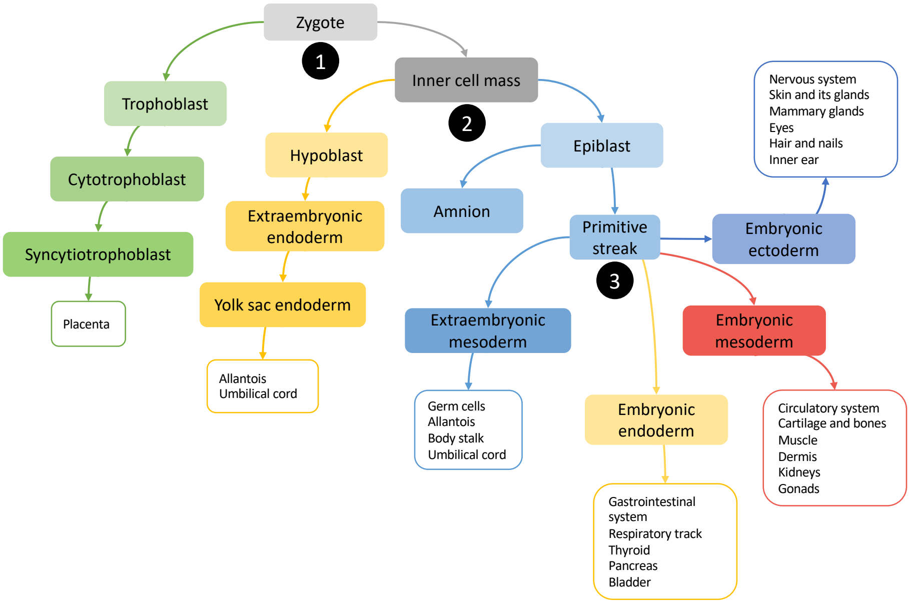


Figure 13. Developmental lineages. Flow chart of cell differentiation and commitment with lineages colored similarly to previous images. Determinant lineage splits are labelled with white numbers in black circles.

3.2 Cell genealogy vs developmental lineage

Whether the cell differentiation tree matches the cell genealogy tree is of particular importance for the study of somatic mutations. One of the first examples of embryonic cell lineage tracing is that of *Caenorhabditis elegans*, a transparent nematode whose adult body has a fixed number of cells (eutely) at ~1,000. Sulston et al. traced the complete development of *C. elegans* and recorded the correspondence between genealogy and final tissue commitment for the different lineages. They noted that despite the fixed relationship between cell ancestry and cell fate, the correlation between the two lacked an obvious pattern (Sulston et al. 1983). For example, they observed that most main lineages contributed both to the generation of muscle and to the generation of neurons, even if in different proportions. Therefore, the total set of neurons was constituted by diverse proportions of cells coming from multiple lineages, and those same lineages were present in muscle, although in different proportions than neurons.

Cell genealogy is much harder to ascertain in mammals, but new techniques such as genome editing of synthetic target arrays for lineage tracing (GESTALT) (McKenna et al. 2016), are leading the way towards acquiring this knowledge. In this case, the barcode is an array of clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 target sites. Random changes are introduced at each cell division, so that the sequencing of single cells allows the reconstruction of their genealogy. However, the resolution is limited to a few divisions.

Nonetheless, studying somatic mutations in humans has shown results consistent with those in *C. elegans*. Lodato et al. showed that a few neurons from the cerebral cortex of normal individuals share somatic SNVs with cardiomyocytes and not with some of the surrounding neurons, indicating they share a more recent common ancestor with those cardiomyocytes (Lodato et al. 2015). They showed that variants produced during embryonic development, with a frequency in neurons (an ectodermal derivative) as low as 2% were also present in tissues derived from a different germ layer, the mesoderm, such as heart and liver.

The relationship between the genealogy and developmental trees was discussed in Arendt et al. They proposed the concept of “serial sister cell types” (Fig. 14). This concept builds upon the observation of body regionalization in metazoans and hypothesizes that cell types arise in the different regions concurrently, producing a lack of correspondence between the trees (Arendt et al. 2016).

Currently, the consensus is that the first commitment between trophoblast and inner cell mass (Fig. 13 (1)) is not random, and that not all cells contribute to both lineages (Zernicka-Goetz, Morris, and Bruce 2009). In contrast, the second fundamental differentiation (Fig. 13 (2)), that between hypoblast and epiblast, is stochastically driven by NANOG and GATA6 (Schrode et al. 2014). However, how subsequent lineage commitments are determined, and which cells differentiate into them is not clear. Gastrulation occurs approximately after the 12th cell division (Snow 1977). Because cell cycles are short at gastrulation, just over 2 hours, the repair machinery efficiency is more limited in that period (Anderson, Lewellyn, and Maller 1997), leading to the appearance of variants that will be present in all the descendant cells. Hence, over 4,000 cells exist at gastrulation, meaning that variants with a frequency as low as 0.02% in the adult could potentially be inherited by cells migrating to all the different germ layers.

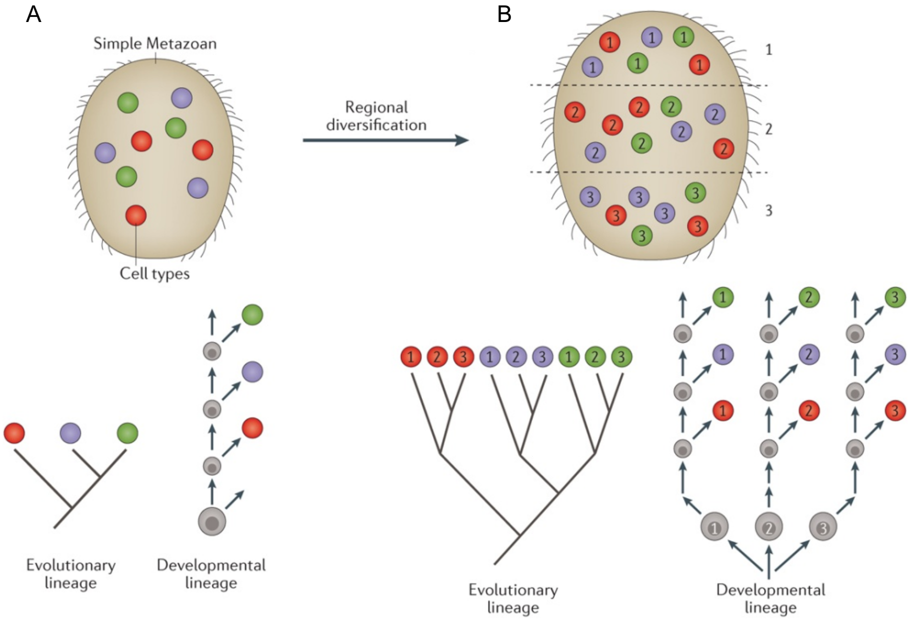


Figure 14. Serial sister cell types. The interrelationship of developmental and evolutionary cell type lineages. **A.** Ancestral state. In a hypothetical simple metazoan, cell types arise from a stem cell-like developmental lineage. **B.** Derived state. Cells first diversify regionally, giving rise to region-specific serial sister cell types. Within each region, cell types arise in parallel, so that developmental and evolutionary lineages differ. (From Arendt et al. 2016)

It has been estimated that ~40% of embryos end in spontaneous abortions, 80% of them before the pregnancy is detected, also known as preclinical losses (Opitz 1987; Wilcox et al. 1988). This is most usually the result of cytogenetic alterations such as aneuploidies and trisomies (H. P. Robinson 1975; M. Ohno, Maeda, and Matsunobu 1991; Minelli et al. 1993) with better formed and latter loss embryos having conditions more compatible with life, such as chromosome 13, 18, 21 or sex chromosome abnormalities (Hardy and Hardy 2015), including somatic alterations (Vorsanova et al. 2005; Lebedev 2011), which indicates how mutagenic the first cell cycles can be.

4. Germline variants

In a diploid organism, there are two copies of each chromosome, the maternal and the paternal. Variants present in the germline of the parents, which are therefore inherited by all the cells of the offspring, are called germline variants. These are the variants usually considered when the genetic variation of an individual is studied.

The main types of variants are single nucleotide variants (SNVs), a nucleotide substitution at a specific genomic position; short insertions or deletions (indels) and structural variation, which includes copy-number variants (larger insertions or deletions), inversions and translocations. When SNVs reach a certain frequency level in a population, usually established at 1% (Cavalli-Sforza and Bodmer 1971), they are considered single nucleotide polymorphisms (SNPs). Different versions of the same locus are called alleles.

4.1 Monogenic variants

Although the epigenome and transcription and translation regulation modulate and tune the genotype to give rise to the phenotype, genetic variants can still directly cause phenotypic variation. Traits can be determined by one or more loci. Those driven by a single gene are called monogenic or mendelian traits, after Gregor Mendel, who proposed the laws of inheritance in the late XIX century.

Mendelian traits can be of dominant or recessive inheritance. Dominant variants are those that cause the phenotype when just one allele is affected. This is the case of the most common variants that confer lactose persistence in humans; individuals with just one allele inducing lactase expression after infancy produce enough enzyme for lactose digestion (Flatz 1984). On the other hand, when the phenotype appears only if both alleles carry the mutation, variants are of recessive inheritance, or haploinsufficient. Examples include diseases such as cystic fibrosis, caused by mutations in the *CFTR* gene (Riordan et al. 1989). In such cases, a single defective allele is not enough to cause the disease, so individuals with one copy are unaffected and are known as carriers.

Both dominant and recessive mendelian mutations have been found to cause neurodegenerative diseases, such as mutations in the alpha synuclein gene (Polymeropoulos et al. 1997) causing dominant inheritance of Parkinson disease

(PD), variants on the Parkin gene (Kitada et al. 1998) causing recessive PD or amyloid precursor protein mutations causing dominant Alzheimer disease (Tanzi et al. 1987).

However, traits can still be monogenic but non-mendelian. Incomplete dominance occurs when the heterozygote phenotype is in between those of the homozygotes. For example, achondroplasia patients present a much more severe phenotype when they are homozygotes, and for most diseases, homozygotes are so rare that whether they have complete or incomplete dominance is unknown (Rimoin, Pyeritz, and Korf 2019). Also, codominance happens when both alleles are expressed independently, such as in human ABO blood groups.

4.2 Polygenic variants

On the other hand, phenotypes determined by more than one locus are known as polygenic traits, such as skin pigmentation in humans, for which more than a dozen loci have been described (Deng and Xu 2018). In the simplest model, the effect of each variant is additive. However, because protein interplay is structured as networks, interactions usually exist between genes in the production of phenotypes, a phenomenon called epistasis. Many phenotypes are very complex, with multiple genes and their interactions involved in their determination (Botstein and Risch 2003). Especially, this is the case for complex diseases; variants causing monogenic or simpler inheritance diseases suffer a stronger negative selective pressure and their frequency lowers in the population. Their appearance is many times the result of genomic instability causing recurrent mutations (Gu, Zhang, and Lupski 2008). However, when a disease is caused by a large number of variants as well as their interactions, the selective pressure on each of them decreases substantially, making it more difficult for natural selection to purge them from populations, especially those with low effective population size.

The heritability of a trait – the proportion of variation that is attributable to genetic factors – can be estimated by calculating the concordance rate between monozygotic twins compared to fraternal twins. Assuming both pairs of twins share the same environment, when identical twins have more similar phenotypes, the variance dependent on genes can be inferred (Sahu and Prasuna 2016). A typical example of a complex quantitative human trait is height. Its heritability has been estimated to be ~80% (Visscher, Hill, and Wray 2008). Genome-Wide Association Studies (GWAS) have been performed to find the variants behind it. In this type of analysis, the association of SNPs and traits is measured by comparing carriers and non-carriers, or in this particular case, people above and below a selected height. The first GWASs on height found only a few associated SNPs, explaining at most less than 4% of the variance (Gudbjartsson et al. 2008). This led to the debate on the missing heritability and where it could reside (Maher 2008). Because of the sizable number of variants involved in the phenotype, only very large samples provide enough power to discover a high proportion of the variance. Polygenic scores are used in this context; they aggregate the effects of many SNPs, even if their independent association on the trait is not significant. When analyzing ~700,000 individuals, a polygenic score could explain ~35% of the variance (Yengo et al. 2018).

However, many times shared environment and especially assortative mating are not properly modelled when estimating heritability (Lynch and Walsh 1998) and association (Marchini et al. 2004). Non-random mating creates population structure, or non-homogeneous populations. If a phenotype varies between populations and subpopulations, this creates spurious correlations between that trait and the variants involved in the structure, especially when using methods that rely on large numbers of small effects, such as polygenic scores (Barton, Hermisson, and Nordborg 2019). Indeed, the latest analyses on polygenic scores for human height found uncorrected population structure caused an overestimation of polygenic scores, which were found to not be easily portable among populations (Sohail et al. 2019; Berg et al. 2019), highlighting methods for correcting for population stratification in GWAS may not always be sufficient for polygenic trait analyses and that any claims of differences in polygenic scores between populations should be treated with caution.

4.3 Copy number variants

The use of microarrays and the expanded application of paired-end sequencing enabled the analysis of small structural variation. Insertions and deletions of regions a few hundred to millions of base pairs, known as copy number variants (CNVs) have been linked to disease (reviewed in Stankiewicz and Lupski 2010) and are variants that also contribute to the evolution of species.

The most common mechanism for a CNV to influence a phenotype is by dosage effect. One of the most famous cases is the gain of amylase copies in the human lineage as an adaptation to a starch-rich diet. The number of copies of the gene an individual has correlates with the concentration the enzyme has on their saliva as well as with dietary starch consumption across populations (Perry et al. 2007).

The dosage effect also produces diseases, such as Charcot-Marie-Tooth disease type 1 (CMT1), the most common inherited peripheral neuropathy. It results from the duplication of the gene *PMP22*, which is part of myelin, the insulation layer surrounding neuronal axons. Myelin formation requires the equilibrium of its components, so the duplication impairs the process (Nobbio et al. 2004), resulting in deficient myelinization, which in turn reduces nerve conduction velocities (Lupski et al. 1992). Because 76–90% of sporadic CMT1 cases have a *de novo* duplication (Hoogendijk et al. 1992; Nelis et al. 1996), it can be inferred that genomic rearrangements in this region are highly recurrent (Lupski 2007). These recurrent duplications are mediated by nonallelic homologous recombination (NAHR) between segmental duplications (Fig. 8) flanking the genomic region containing this gene (Inoue et al. 2001; Yuan et al. 2015). Also, deletions of this same region have been linked to a different disease, hereditary neuropathy with liability to pressure palsies (Inoue et al. 2001).

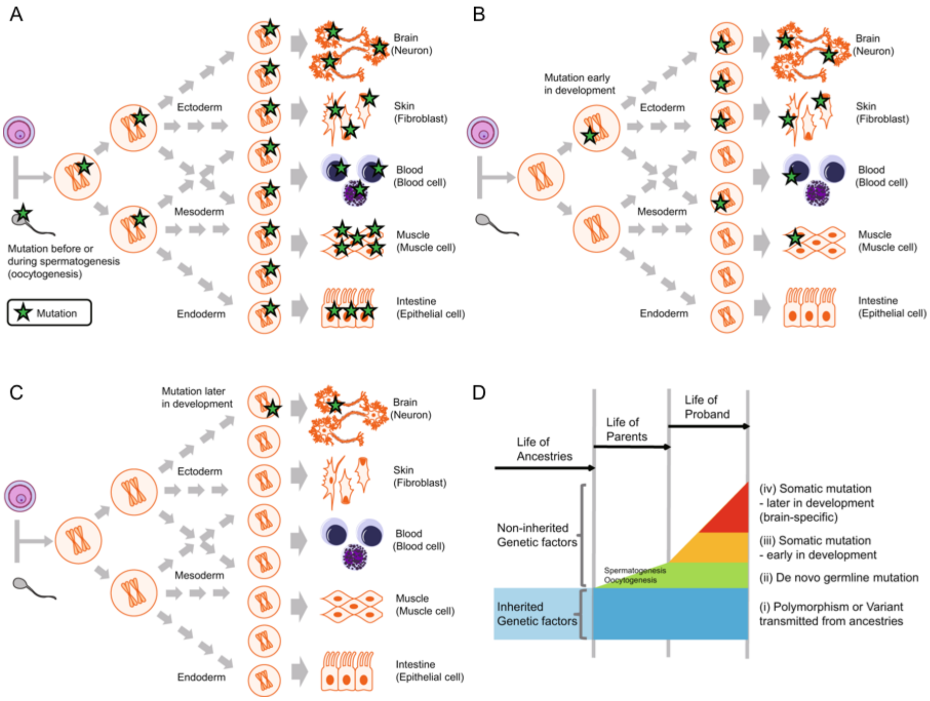
Besides dosage effect, the deletion of a region can cause disease by unmasking recessive mutations or functional polymorphisms of the remaining allele, as observed for Sotos syndrome (Kurotaki et al. 2005). Also, smaller deletions, affecting portions of protein coding genes can result in abnormal proteins, lacking functional domains (Licht et al. 2006).

However, not only coding region CNVs can cause disease, the deletion of regulatory regions can also produce pathologies. Nonsense, missense and frameshift mutations in *FOXL2* result in blepharophimosis-ptosis-epicanthus inversus syndrome (BPES) (Beysen, De Paepe, and De Baere 2009). *FOXL2* is a developmental gene with a strictly regulated spatiotemporal expression pattern. Microdeletions upstream and downstream of the gene were found in 4% of BPES cases, and chromosome conformation capture of the region revealed physical interactions with *FOXL2* promoter (D'haene et al. 2009), explaining the appearance of the condition. Also, analysis of deletions common in Van Buchem syndrome patients helped to identify that sclerostin was involved in the disease, since the deleted regions affected regulatory elements of that gene (Loots et al. 2005).

As discussed in the previous section, cytogenetic alterations are very common in early embryos, including aneuploidies and trisomies as well as smaller rearrangements. The best tolerated trisomy is that of chromosome 21, Down syndrome (DS). It has been found that proteins in this chromosome interact much less between them than those of other chromosomes, explaining the high viability of DS patients (Kirk et al. 2017). For the same reason, the consequences of gene duplications are very diverse depending on where in the interactome they are located. For example, alpha synuclein duplications have been described in Parkinson disease (Singleton et al. 2003), while the DS trisomy of 21 causes the overexpression of the *APP* gene, giving rise to the Alzheimer pathology in DS patients.

5. Somatic mutations

Mutations that appear during embryonic development or adult tissue maintenance and are therefore present only in a proportion of the cells in an individual are termed somatic mutations. They are also known as postzygotic variants, owing to the fact that they occur after the formation of the zygote. Depending on the developmental stage when the mutation occurs, it will affect a varying proportion of cells and tissues (Fig. 15), including the individual's germline. The resulting presence of cells with slightly different genomes in a single individual is known as mosaicism.



A
Mutation before or during spermatogenesis (oocytogenesis)
Mutation
Ectoderm
Mesoderm
Endoderm
Brain (Neuron)
Skin (Fibroblast)
Blood (Blood cell)
Muscle (Muscle cell)
Intestine (Epithelial cell)
B
Mutation early in development
Ectoderm
Mesoderm
Endoderm
Brain (Neuron)
Skin (Fibroblast)
Blood (Blood cell)
Muscle (Muscle cell)
Intestine (Epithelial cell)
C
Mutation later in development
Ectoderm
Mesoderm
Endoderm
Brain (Neuron)
Skin (Fibroblast)
Blood (Blood cell)
Muscle (Muscle cell)
Intestine (Epithelial cell)
D
Life of Ancestries
Life of Parents
Life of Proband
Non-inherited Genetic factors
Inherited Genetic factors
Spermatogenesis Oocytogenesis
(iv) Somatic mutation - later in development (brain-specific)
(iii) Somatic mutation - early in development
(ii) De novo germline mutation
(i) Polymorphism or Variant transmitted from ancestries

Figure 15. *De novo* and somatic mutations. A. *De novo* mutations occur at some point in the cell lineage of parental germ cells. They are present in the zygote and therefore in all tissues and cells of the offspring. B. Early somatic mutations occur during the first cell divisions of the embryo and because the mutant cells contribute to multiple tissues, even from different germ layers, they are spread in the individual. C. Later somatic mutations are confined to a smaller cell lineage and are only present in some tissues of the individual. D. Appearance of the different types of mutations. (i) Blue variants are those that occurred more than a generation ago, germline inherited variants or polymorphisms. (ii) *De novo* mutations, in green, appeared at some point during parental development or tissue maintenance, the specific moment determines the amount of germ cells carrying the variant and is fundamental for estimating recurrence probabilities. (iii and iv) Early (orange) and late (red) somatic mutations occur during embryonic development. (From Nishioka et al. 2018)

5.1 First observations

Mosaicism was observed early on as a result of chemical or less frequently, radioactive mutagenesis in lab animals producing striking mosaics (reviewed in Auerbach and Kilbey 1971). In 1961, Mary Lyon showed that half the human population is functionally mosaic for chromosome X (Lyon 1961). Soon thereafter Gartler and Francke proposed half chromatid or early somatic mutations as a mechanism for the appearance of Lesch-Nyhan syndrome (Gartler and Francke 1975). This impairing X-linked recessive condition is caused by a deficiency of the enzyme hypoxanthine-guanine-phosphoribosyl transferase (HGPRT) and produces hyperuricemia, which results toxic to the central nervous system. Given that male patients are not able to produce offspring, the disease should diminish in frequency. However, new mutations seemed to occur with enough frequency to maintain the observed rate. Further, male patients with homozygous unaffected mothers were observed, so somatic mutations were proposed as an explanation for both phenomena. Mosaicism was also suggested to be the cause of other X-linked conditions visible on the skin, such as incontinentia pigmenti (Lenz 1975).

A decade later, Rudolf Happle evidenced that many of these hereditary conditions manifested on the skin following the lines of Blaschko, patterns previously associated with embryonic development, with a typical dorsal V-shape and an abdominal S-figure. He proposed that these lines observed in women affected with X-linked skin disorders could result from the clonal proliferation of two functionally different populations of cells during early embryogenesis of the skin, each with a different inactivated X chromosome (Happle 1985). Interestingly, he found the same skin pattern accompanied a different disease, the McCune-Albright syndrome, which was completely sporadic. Since the Blaschko lines suggested the existence of a distinct cell population carrying the mutation during skin development, but no hereditary cases had been reported, he suggested that the causal mutation had to be lethal to embryonic development and only compatible with life when in a mosaic state (Happle 1986a). This supposed the description of the first obligate somatic disease, a concept that was then extended to many others, such as the well-known Proteus syndrome (Happle 1986b; Clark et al. 1987).

5.2 Cancer

Cancer as a model for understanding somatic mutations

Cancer could be considered as the most prevalent disease caused by somatic mutations. In the 1920s, it was proposed that tumors originate from cells affected by genetic mutations. This was inferred from the observation of chromosomal aberrations in cancer cells together with the fact that contact with mutagenic agents increased the risk of cancer (Nordling 1953). In the following decades, it became clear that the age-of-onset distribution suggested that two hits or even multiple hits were necessary for cancer development: at least one mutation to increase cell division rate and a second one to release the cells from control (Armitage and Doll 1957; Ashley 1969). Later, it was proposed that both sporadic and inherited forms of retinoblastoma could be caused by lesions to the same gene, the so-called “two-hit hypothesis” (Knudson and Jr. 1971), with one inherited germline mutation being the first hit and the second one occurring during somatic development. This theory was corroborated when the responsible gene was found (Friend et al. 1986) and it was confirmed that a mutation in each allele produced the total loss of function of the retinoblastoma protein gene, a cell cycle regulator and the first tumor suppressor gene to be discovered. This finding led to the search for genes whose mutation could cause different types of cancers, an effort that caused the discovery of multiple cancer driver genes (M. H. Bailey et al. 2018).

The advent of DNA sequencing and the fast reduction of its cost paved the way towards understanding the origin of somatic mutations in cancer genomes. The sequencing and comparison of melanoma and lymphoblastoid cell lines from the same patient showed that almost two thirds of the mutations were C>T transitions (Pleasance, Cheetham, et al. 2010), which were already known to be produced by UVB light mutagenesis on dipyrimidines containing 5-methylcytosine (Gerd P. Pfeifer, You, and Besaratinia 2005). Similarly, the comparative analysis of a lung tumor genome from the type of cancer most associated with smoking showed the high prevalence of G>T transversions (Pleasance, Stephens, et al. 2010). This was consistent with the pattern observed after exposure to tobacco carcinogens, polycyclic aromatic hydrocarbons, that covalently bond to guanines in the GpA context (Denissenko et al. 1996). These discoveries showing that different carcinogens produce different mutational patterns in a context dependent manner led to the development of mutational signature analysis, which focuses on the deconvolution of the different patterns (Alexandrov et al. 2013a). Mutational signature analysis of over 7,000 cancers showed the existence of at least 21 signatures, with signature 1 being ubiquitous (Alexandrov et al. 2013b). This signature is characterized by C>T transitions caused by the spontaneous deamination of methylated cytosine, which occurs predominantly at NpCpG trinucleotides (G P Pfeifer 2006). Although it was discovered in disease, this is a universal process, so it has been observed in both *de novo* (Rahbari et al. 2016) and somatic mutations (Bae et al. 2018) as well as in population variation studies (Mathieson and Reich 2017).

This shows the potential cancer genome analysis has to discover universal patterns and mechanisms of somatic mutation. In fact, it has been shown that the mutation rate along the genome is not as homogeneous as once thought, with density of mutations at the 1-Mb scale being very variable and similar among different cancer genomes as well as the germline. Mutation rates correlate with GC content, replication timing, nucleosome occupancy (Hodgkinson, Chen, and Eyre-Walker 2012), transcribed regions (Pleasance, et al. 2010) and euchromatin (Schuster-Böckler and Lehner 2012). As fundamental repair machinery is sometimes inactivated in cancer, it poses an exceptional context to find the mechanisms behind this variability. MMR deficient tumors show a considerably flatter mutation rate variability, demonstrating the relevance this pathway has in generating this variation (Supek and Lehner 2015). MMR machinery is recruited by the histone modification H3K36me3 (Supek and Lehner 2017), which has a differential presence in introns than exons, explaining the lower mutation rate of the latter (Frigola et al. 2017).

Differences with other somatic mutation diseases

When compared to other somatic mutation diseases, cancer is a different scenario. Developmental somatic mutation diseases such as Proteus syndrome (Happle 1986b) are caused by mutations that do not confer clonal advantage to the mutant cells. That is, as they do not divide faster than other cells, mutant cells do not outgrow other cell lineages, so their proportion in the tissues is a product of drift rather than selection. On the contrary, since cancer is caused by the uncontrolled division and migration of cells, the responsible mutations are precisely those that give the mutant cells a proliferative advantage and therefore their proportion increases driven by positive selection (Bignell et al. 2010).

This conceptual difference implies significant technical changes in their analysis. In cancer, clonal expansion produces enough mutant cells to be sequenced in bulk. Also, the use of paired samples can help in identifying driver mutations – those only present in the tumor and responsible for its malignancy – although passenger mutations are also present in the tumor (Haber and Settleman 2007). In other diseases, mutation frequencies can vary substantially. Considering that embryonic cells contribute to the extraembryonic tissues (Fig. 13) and can do so in an asymmetrical way (Ju et al. 2017), a somatic variant could putatively be present in more than 50% of the cells. On the other hand, a mutation occurring later could be very rare in the tissues (Fig. 15). Even if the former is the case, a mutation present in most tissues can still be responsible for a disease if the affected gene is only expressed or relevant for a specific organ. Further, because approximately 12 cell divisions occur before gastrulation (Snow 1977), even mutations present in only 0.02% of the cells can be found in different tissues from all germ layers. For this reason, sequencing the affected organ and comparing it to an unaffected tissue is an inadequate strategy, because for a reasonable sequencing resolution, variants will most probably be present in both. This suggests that variant calling algorithms should be quite different for cancer and other somatic mutation diseases. In particular, systematic sequencing errors or those arising from misaligned unresolved regions of the genome become relevant, since they produce false positives for different samples in a similar proportion. Yet, in the worst-case scenario in cancer calling, such artefacts would be considered as shared between control and tumor DNA and therefore discarded.

These systematic or recurrent errors are often only distinguishable from true somatic variants by the fact that they are found in multiple tissues and individuals, which given the somatic mutation rate found to date (Lodato et al. 2015; Alexej Abyzov et al. 2017; Bae et al. 2018) is very unlikely to be the case. The probability of those calls being biased sequencing errors or other kind of artefacts is much higher. Nonetheless, the interaction of DNA with certain local chromatin features has a strong influence on how nucleotides are damaged and repaired at the local level, which ultimately results in different mutation probabilities along the genome (Gonzalez-Perez, Sabarinathan, and Lopez-Bigas 2019). Moreover, when analyzing patients suffering from the same disease, some recurrent events could be expected. This is why comparisons with a panel of controls can be helpful to discern between these scenarios.

Cancer driver somatic mutations in healthy tissues

Nonetheless, it is becoming clear that even for assessing the relevance of mutations in cancer, basic knowledge on healthy tissue development and maintenance and the forces shaping clone dynamics is necessary. In one of the first examples of healthy tissue analysis, Martincorena et al. sequenced 74 cancer genes at an average depth of coverage of 500x in 234 biopsies of sun-exposed but physiologically normal skin from four individuals. They found a surprisingly high burden of mutations, higher than that of many tumors, some of them already under strong positive selection. Hundreds of evolving clones per square centimeter of skin and thousands of mutations per skin cell were detected. They suggested that there may be an underlying reservoir of competing clones in normal skin. It was also noted that even if a drug that killed all cells with an inactivated tumor suppressor gene commonly inactivated in squamous cell carcinomas – *NOTCH1* – was developed, 60% of the tumors could be treated but with considerable collateral damage to physiologically normal skin, whose cells frequently carry inactivated *NOTCH1* (Martincorena et al. 2015).

To test whether these observations were the result of skin exposure to mutagens like UV light, a similar study was carried out on 844 small samples of normal esophageal epithelium from nine individuals. Again, 74 cancer genes were target sequenced at high depth, 870x. Clones carrying mutations in 14 of them were found to be under strong positive selection. Interestingly, mutations in *NOTCH1* were more common in normal esophageal epithelium than in esophageal cancer, demonstrating that the appearance of these genes in cancer could be caused by their high mutation frequency in the normal cells from which tumors evolve (Martincorena et al. 2018). On the other hand, CNVs were infrequent, suggesting negative selection. A similar observation has been recently made for neurons (Chronister et al. 2019).

Two recent preprints on colorectal and endometrial epithelium came to similar conclusions. Somatic mutations on cancer driver genes were present in ~1% of normal colorectal crypts in middle-aged individuals, indicating that adenomas and carcinomas are rare outcomes of a pervasive process of neoplastic change across morphologically normal colorectal epithelium. However, the structure of this tissue, organized in crypts, constrained clonal expansion (Lee-Six, Ellis, et al. 2018). In contrast, a vast majority and even all of the endometrial glands were found to be colonized by cells carrying driver mutations in most women, probably because periodic endometrial shedding creates more opportunities for such clones to expand. Further, mutational burdens increased with age as well as with other factors such as body mass index and parity (Moore et al. 2018).

All of these studies were performed on epithelium, a tissue with an elevated turnover in which mutations conferring proliferative advantages would be expected to spread with age, even if differences are observed depending on tissue structure and renewal. Also, many of the studies only sequenced cancer driver genes to a sufficient coverage, which limits their ability to study population dynamics in these healthy tissues.

Analyzing a different tissue, whole-genome sequences of normal blood from 241 adults at a coverage of ~30x resulted in the identification of 163 early embryonic mutations. A few of them were also found in breast, an ectodermal tissue, including some variants whose frequencies were smaller than 5% in both tissues. Reconstruction of cell lineages with the identified mutations suggested that the two daughter cells of many early embryonic divisions contribute asymmetrically to adult blood at an approximately 2:1 ratio (Ju et al. 2017). This could be general to other adult tissues, but since blood development and maintenance is clonal (Sun et al. 2014), it might well be an exception. Indeed, in the immune system, developmentally programmed somatic mutations produce cellular diversity for antigen recognition through V(D)J recombination, the process by which T and B cells randomly assemble different gene segments to construct varied lymphocytes receptors during early development (reviewed in Alt et al. 1992). Furthermore, in response to antigen, somatic hypermutation occurs, which involves the MMR machinery (Chaudhuri, Khuong, and Alt 2004), making blood a tissue where somatic mutations are expected to be at higher rates.

5.3 Somatic mutations in healthy tissues

Rehen et al. showed that somatic copy number variants as big as chromosome 21 aneuploidy were frequent in the healthy human brain. Later, it was also shown that retrotransposons mobilize during neurogenesis creating mosaicism both in both rodents and humans and (Muotri and Gage 2006; Coufal et al. 2009; Muotri et al. 2009; Singer et al. 2010), indicating this process is common in the mammalian development. In the past five years, several studies have tried to characterize the number of somatic mutations in human cells, adult and fetal, from different tissues.

In a recent study, the whole genomes of 36 single neurons from the prefrontal cortex of neurotypical individuals were sequenced. The somatic SNVs reflected a pattern of transcription damage rather than replication and the cell tree reconstructed with the variants evidenced the existence of different lineages. Recurrent SNVs in neurons were rare – 1 to 11 per neuron – compared to a higher number of potentially unique SNVs – 300 to 900 – which are difficult to reliably distinguish from errors produced by amplification methods. Interestingly, some SNVs exclusive to one neuron lineage were also present in cardiomyocytes, even though they arose from a different germ layer, the mesoderm (Lodato et al. 2015). This is not completely unexpected since those variants were present in more than 2% of the cells in both tissues, pointing towards them happening at the 6th division. Significant proliferation and cell movements occur between then and gastrulation during embryonic development, providing an explanation for their presence in multiple tissues. Similar numbers of variants and proportion of shared mutations were found using mice reprogrammed adult postmitotic neurons generated by somatic cell nuclear transfer of neuronal nuclei into enucleated oocytes (Hazen et al. 2016).

A different technique was used to explore somatic mutations in fibroblasts derived from children. Human induced pluripotent stem cell lines were derived from 32 fibroblasts. This technique uses clonal expansion of the cells to amplify the original DNA up to a point where it can be sequenced without PCR amplification. Then, variants present in the original fibroblast must be present in all cells, or 50% of the reads in bulk sequencing, while variants arisen during culture will be less frequent. On average, each fibroblast carried ~1,000 mosaic SNVs. This number was similar in adults, indicating that somatic mutations happen mainly during embryonic development (Alexej Abyzov et al. 2017).

Sequencing clonally expanded forebrain neurons from fetuses, as expected, a lower number of variants was found, 200-400 SNVs per cell. SNVs with a frequency higher than 2% in brain were also present in the spleen (Fig. 16), revealing a pregastrulation origin. Assigning mutations to the first five postzygotic cleavages based on their frequency showed an early mutation rate of ~1.3 mutations per division per cell, with a mutational spectrum similar to that of *de novo* mutations. However, variants assigned to later divisions, during neurogenesis, implied a higher rate and a spectrum associated with oxidative damage, which the authors proposed to be a result of the development of the cardiovascular system (Bae et al. 2018). The inferred somatic mutation rate is higher than that found in adult epithelium (Lee-Six, Øbro, et al. 2018; L. Moore et al. 2018), once again indicating higher mutation rates during embryonic development.

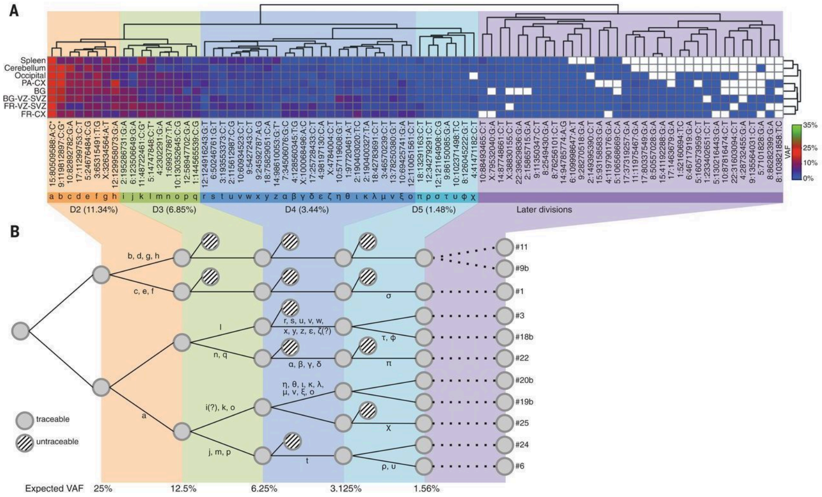


Figure 16. Assignment of somatic mutations to early development divisions. A. Hierarchical clustering of SNVs genotyped in the different brain regions and spleen by their variant allele frequencies (VAFs). White squares represent zero VAF. **B.** Reconstructed cell genealogy tree and assignment of SNVs. Conflicts of SNV assignment are denoted by "?". Expected VAF denotes the VAF mutations arising at each stage should have, assuming equal contribution of all lineages to tissues. (From Bae et al. 2018)

5.4 Somatic mutations can cause disease

Most of the early observations linking somatic mutations with disease were cases of parental mosaicism. Maternal mosaicism for chromosome 21 trisomy was reported in the early 1960s (Weinstein and Warkany 1963) and during the 1980s multiple studies showed germline mosaicism in phenotypically normal parents could affect recurrence risk of diseases such as achondroplasia or Duchenne muscular dystrophy (reviewed in Hall 1988) or retinoblastoma (Sippel et al. 1998).

The first decade of this century witnessed the association of somatic mutations to neurological diseases. Gleeson et al. discovered over 30% of cells had to be mutant for the *DCX* gene for patients to develop doublecortex and lissencephaly. It was also shown that microdeletions of *NF1* produce neurofibromatosis type-1 (Messiaen et al. 2011) and *AKT3* mosaicism contributes to hemimegaloencephaly (Poduri et al. 2012).

Examples of somatic mutations causing diseases have become increasingly common over the past lustrum. Priest et al. were among the first to show the relevance low frequency somatic mutations can have. An infant with perinatal long-QT syndrome (LQTS), a life-threatening arrhythmia, carried an SNV in a sodium channel, *SCN5A*, in 8% of their leukocytes. The same variant could be detected in 5.4% and 11.8% of cardiac transcripts in two ventricular myocardial samples. A computational model showed the delay of sodium current caused by the mutation was sufficient to explain the arrhythmia. It was also found that a small proportion of LQTS patients (13/7,500 or 0.17%) also carried early somatic mutations (Priest et al. 2016).

A second case highlights the relevance of low frequency variants in a phenotypic rescue event. Hutchinson-Gilford progeria syndrome (HGPS) is a fatal sporadic dominant condition in which mutations of the *LMNA* gene cause premature ageing. Patients heterozygous for a T>A mutation at a specific position suffer with very severe cases of progeria. On the other hand, individuals with a T>C at the same position present milder symptoms. A case with 4.7% of cells carrying T>C and 41.3% carrying T>A was described, and a significantly milder phenotype than the one found with just T>A was observed. The authors' hypothesis was that the T>A variant was a germline *de novo* mutation and the A>C substitution occurred during embryonic development, partially rescuing the phenotype (Bar et al. 2017).

Primary immunodeficiencies are oftentimes caused by germline *de novo* mutations in a group of well-characterized genes. A target sequencing study showed that roughly 25% of genetically undiagnosed cases could be explained by a somatic mutation in one of these genes, with mutant cell frequencies ranging from 0.8% to 40.5% (Mensa-Vilaró et al. 2019).

Neurological developmental diseases have also been linked to somatic variants. A case of hemimegaloencephaly was found to be caused by a somatic mutation in *AKT3* in 35% of brain cells and not detected in blood at a 2% resolution (Poduri et al. 2012). Also, focal cortical dysplasia type II has been found to be the result of somatic mutations in *MTOR* in 6-13% of brain cells and undetectable in 600x blood exome sequencing (Lim et al. 2015; Park et al. 2018). Another study identified nine somatic variants in early-onset Alzheimer disease patients in genes related to the disease: two in *APP*, five in *SORL1*, one in *NCSTN*, and one in *MARK4* with allele fractions ranging from 0.2% to 10.8% (Nicolas et al. 2018).

Further, somatic mutations can be used to associate genes with diseases in discordant monozygotic twins. This helped to detect new as well as previously described genes involved in amyotrophic lateral sclerosis, schizophrenia, Tourette's syndrome and autism spectrum disorder (Vadgama et al. 2019).

Finally, the burden of somatic variants was found to be much higher in patients with autism compared with controls (Lim et al. 2017; Dou et al. 2017), indicating the putative relevance of somatic mutations in complex diseases.

Somatic mutations and ageing

Somatic mutations were proposed to be implicated in ageing more than two decades ago, in the somatic mutation theory of ageing. It suggests that ageing did not evolve but has always been present, occurring as the result of fundamental chemical processes (Morley 1995). In fact, somatic SNVs accumulate with age in the brain (Lodato et al. 2018). Also, the absence of negative selection in cancer and on point mutations during normal somatic tissue maintenance suggests that even point mutations deleterious to the carrying cell do not drive cellular senescence, exhaustion, and death (Martincorena et al. 2017). This suggests that mutations could accumulate in the tissues with age and be the cause behind the structural and functional changes that accompany the passage of age.

All these studies illustrate how little we know about somatic evolution within healthy tissues, a fundamental process that is likely to take place to varying degrees in every tissue of every species (Martincorena et al. 2018), with consequences in disease and ageing. It can be considered that all germline variants, including population polymorphisms, were at some point *de novo* mutations, which in turn were somatic mutations that occurred in the germline lineage of an individual (Fig. 15). Therefore, the study of somatic mutation emergence, and the molecular mechanisms behind this process is also the foundation towards understanding the origin of population variants and probably to resolve the issue of the molecular clock in population genetics. Even if the germline is a privileged cell lineage with strong selective pressure against deleterious mutations, there is still controversy on how germline mutations originate. Contradicting evidence pointing towards the main role of either replication errors (Jónsson et al. 2017) or DNA damage (Z. Gao et al. 2018) is still being leveraged. Understanding the determinants of somatic mutation appearance can elucidate some of these questions.

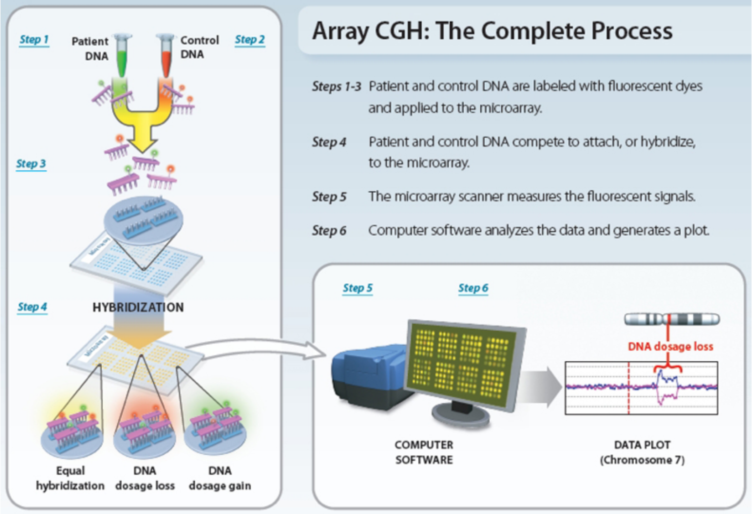
6. Genome analysis technologies

Since the discovery of the molecular structure of the DNA, several tools have been developed to analyze it, so that its changes can be linked to phenotypes and diseases. Here we focus on the methods relevant for this thesis.

6.1 Comparative genomic hybridization arrays

Comparative genomic hybridization (CGH) consists of the simultaneous hybridization of differentially labelled test and reference DNA to genomic probes. Duplications or deletions are then identified as differences in the ratio of the fluorescent labels. Traditional CGH was developed as a method for achieving higher resolution than other forms of cytogenetic analysis such as Giemsa banding, which could only detect events affecting heterochromatin distribution pattern. It was first applied using whole metaphase chromosomes on a slide as probes for cancer samples, which allowed the identification of new amplified loci (Kallioniemi et al. 1992).

Later on, array CGH (aCGH) was developed by applying this concept to DNA microarrays – a solid surface containing known nucleotide sequences at specific locations – to detect copy number changes on a genome wide and high-resolution scale (Solinas-Toldo et al. 1997). This is achieved by first attaching 100-200 kb cloned DNA fragments to the array. The test DNA, such as DNA from a patient suspected to have a CNV, is fragmented and labelled with a red fluorophore (cyanine 5), while a control genome is labelled with a green fluorophore (cyanine 3). Equal amounts of both samples are co-hybridized to the array. Then, both fluorescent light intensities are measured at each array spot, so that an excess of red light indicates a duplication of the region containing the probe in the test genome, whereas an excess of green light results from its deletion (Fig. 17). This way, estimations of copy number with respect to the control sample can be calculated with high resolution. However, because DNA must be previously fragmented, balanced chromosomal rearrangements are not detectable, since the translocated pieces will be in the pool of fragments irrespectively of their position in the test genome. Nonetheless, most rearrangements, even if apparently balanced, are associated with deletions whose detection with aCGH can be useful for clinical treatment (Astbury et al. 2004; Schluth-Bolard et al. 2009).


Array CGH: The Complete Process

Steps 1-3 Patient and control DNA are labeled with fluorescent dyes and applied to the microarray.

Step 4 Patient and control DNA compete to attach, or hybridize, to the microarray.

Step 5 The microarray scanner measures the fluorescent signals.

Step 6 Computer software analyzes the data and generates a plot.

Figure 17. Array comparative genomic hybridization process. (From Shaw-Smith 2004)

Since genomic rearrangements occur frequently in cancer, array CGH has been extensively used to identify them. Due to its higher resolution, several tumor suppressor genes located in previously identified loci were found with this technology (Hodgson et al. 2001; Lassmann et al. 2007). Interestingly, the correlation of poor prognosis in mantle cell lymphoma with aCGH data showed that 8p and 13q14 deletions were relevant for survival (Kohlhammer et al. 2004).

Further, small deletions have been associated with other diseases such as autism with the use of aCGH in large patient cohorts (Weiss et al. 2008), even in a somatic state (Celestino-Soper et al. 2011). Several other intellectual impairment syndromes are caused by microdeletions. Previous methods could not identify these short changes, but aCGH has been proven to detect them (Vissers et al. 2003) albeit with a high false positive rate (Sagoo et al. 2009). Also, its use for prenatal diagnosis has been implemented (Rickman et al. 2005), although orthogonal methods such as SNP arrays can be of use to detect triploidy (Wapner et al. 2012).

Finally, cases of *APP* duplication in early onset Alzheimer patients have also been detected with aCGH (Kasuga et al. 2009) as well as multiplications of the α-synuclein gene in familial Parkinson disease patients (Sironi et al. 2010; Ferese et al. 2015).

6.2 DNA sequencing

The first DNA sequencing technologies had a very low throughput. Sanger sequencing requires four separate reactions, each with one different labelled dinucleotide. Primers are hybridized to the input DNA strands and extended with a mixture of the four regular nucleotides and one of the labelled dinucleotides. Each time a base complementary to the dinucleotide of that reaction is present in the input sequence, extension is arrested in a few of the fragments while the others continue until the complementary base appears again. After this process, the resulting fragments are sorted by size in a polyacrylamide gel, each reaction in one lane, so that an autoradiography shows the arrested fragments and hence the nucleotide sequence can be inferred (Sanger, Nicklen, and Coulson 1977). Sequences as long as 1,000 bp can be determined with this method and a physical map was used to order and concatenate the obtained sequences into chromosomes or genomes.

Later on, shotgun sequencing made sequencing possible without the need of a physical map. DNA sequences are randomly broken into fragments called reads, which after sequencing, are assembled into the original sequence by the use of their overlaps to align them. DNA sequences coming from the same genomic region are broken into different reads and the same position is sequenced multiple times. The number of reads a position is sequenced by is known as depth of coverage. As an example, the Human Genome Project, the first to use shotgun sequencing, sequenced most of the human genome at about 10x (International Human Genome Sequencing Consortium 2001).

Next-generation sequencing

Next-generation sequencing, or high-throughput methods, sequence DNA in a different manner, called sequencing-by-synthesis (Fig. 18). The input DNA is broken into small fragments whose ends are then ligated to adaptors, short sequences complementary to flow cell oligos as well as to the sequencing primers. In paired end sequencing, fragments will be sequenced from both ends. Hence, to avoid overlapping, DNA is usually shredded by sonication into fragments with length, or insert size, longer than double the read length.

The flow cell is a glass slide divided in lanes with two type of oligos fixed to it. Fragments attach to the flow cell by one of the adaptor strands. Then, the complementary sequence is formed by polymerase chain reaction (PCR) extension from the adaptor. Once finished, the resulting double stranded molecule is denatured and the original strand is washed away, leaving only the sequence attached to the flow cell. Then, bridge PCR amplification is used to generate clusters. That is, the attached sequence bends over to pair its second adapter to an oligo in the flow cell and polymerase generates the complementary sequence, which is again denatured. This time both sequences are tethered to the flow cell. The process is repeated multiple times in order to get local clonal amplification of the original fragment, or clusters of the same input fragment. Then, the reverse strands are cleaved and the primers complementary to the first sequencing primer, or read 1 primer, are added. Fluorescently tagged nucleotides are added at each cycle, and those complementary to the fragment are paired to it. Upon light stimulation, each newly added nucleotide emits a signal depending on its fluorescent tag. Light wave lengths and intensities are recorded and used to determine the sequences, a process known as base calling. Base qualities are also assigned to each nucleotide (Bentley et al. 2008).

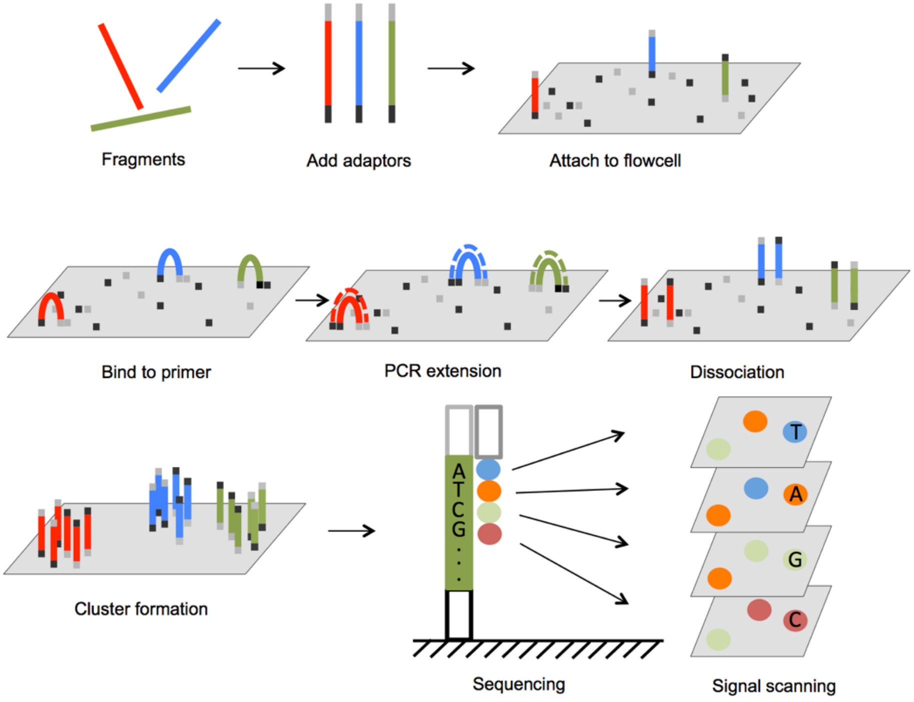


Figure 18. Illumina sequencing process. Input DNA is fragmented, and adaptors and sequencing primers are ligated to each fragment. Fragments are attached to the flow cell by one of the adaptors. Then, clusters of each sequence are formed by bridge amplification. Finally, sequencing-by-synthesis is performed by recording the fluorescent light emitted at each cycle by each cluster. (From Lu et al. 2017)

The number of cycles performed determines read length. After all the cycles are completed, the fragments synthesized during sequencing are washed away. Then, the original fragment bends over, pairs with the second oligo, is extended by polymerase, and the original forward strands are cleaved, leaving only the reverse strands in the flow cell. Finally, the read 2 sequencing primer is added and the process is repeated to sequence the fragment from its other end (Bentley et al. 2008).

It is important to differentiate between strand and read pair. The strand of a sequence is determined only after aligning it to the reference genome. By convention, the reference genome sequence is considered the forward or plus (+) strand, while its reverse complement is considered the reverse or minus (-) strand. As previously described, in paired end sequencing, read 1 is the first read to be sequenced. In Illumina, sequencing starts with the 5' end sequencing primer. Therefore, reads that are read 1 can be forward or reverse, and the same is true for read 2.

Over run time, clusters can grow to the point where they start to overlap. This, together with reagents suffering suboptimal temperatures for several hours or even days during sequencing, implies that base calling from sequencing read 2 is more difficult, hence usually read 2 base qualities are lower.

Calling somatic mutations from next-generation sequencing reads

In order to obtain the input DNA, it has to be extracted from a bulk sample, that is, a piece of tissue. Often, the chosen tissue is blood, since it is accessible without the need of an invasive biopsy. Cell membranes are first broken to release the DNA, which is then precipitated and separated from other cell components. This way, most of the DNA in all the cells present in the sample ends up in the extraction. Then, the extracted DNA is shred into small fragments and a DNA library is prepared by ligating the sequencing adaptors to them. In the end, only a small proportion of those fragments is sequenced, so when the obtained reads are mapped to the reference genome, the different reads overlapping the same region come from different DNA molecules, and most probably, from different cells. Since the main interest of this project are somatic mutations, which will be present only in one of the chromosomes of a small proportion of cells, after library preparation, only some library fragments will contain the mutation, and this number will be proportional to the number of cells carrying it. This way, from the proportion of reads supporting the alternative allele, we can infer the proportion of mutant cells in the tissue.

Traditional somatic mutation callers were developed for cancer and were focused on detecting mutations present in the tumor and absent in the normal tissue, so they usually require paired samples. In fact, most reviews and evaluations of somatic variant callers are centered on the use of paired samples (Krøigård et al. 2016; C. Xu 2018). However, as previously discussed, expectations in a developmental somatic mutation scenario are different. Nonetheless, some callers can be used without matched normal samples, such as Varscan 2 (Koboldt et al. 2012), Mutect2 (Cibulskis et al. 2013) or MosaicHunter (A. Y. Huang et al. 2017). Callers use read features to assess the probability of alternative allele supporting reads being artefactual or true somatic mutations. On the one hand, MosaicHunter combines a Bayesian genotyper with filters that heavily refine calls based on multiple features, including extreme depth, repetitive regions, strand bias or a variant being observed in population databases. On the other hand, Varscan 2 applies heuristic methods to detect variants and can be tuned for different sensitivity levels. Of course, if sensitivity is set high, a multitude of false positive variants is also called. Other callers such as Mutect2 use intermediate strategies.

Somatic mutation calling from a single sample is a complex process with many features flagging different noise sources. Thus, using the consensus of multiple callers has been proposed for increasing accuracy (Goode et al. 2013). This is because certain read features indicate a higher probability of a call being a false positive. However, they cannot perfectly separate true and false positives, so the combination of different callers with a variety of confidence thresholds helps to determine a more reliable set at the cost of sensitivity. The lack of a benchmarking dataset with a known ground truth for somatic mutations hinders the task of developing accurate somatic callers.

6.3 Exome sequencing

Although genome sequencing price decreased almost exponentially in the first decade of this century (Check Hayden 2014), it still costs several hundreds of euros to sequence a genome at the coverage needed for population genetics or the discovery of deleterious germline variants. Somatic mutation calling requires a much higher coverage to have the power necessary to uncover a significant number of variants, usually at least 3 to 4 times more coverage. Also, mutation rates are not high, so it could be argued that it is more probable that *de novo* mutations causing human diseases are located in the exome, where they have a more direct effect on the phenotype. Since the exome is about 1% of the total sequence of the genome, even if capture baits are necessary, the cost of sequencing only this region is reduced proportionally. Accordingly, exome sequencing has been extensively used for the detection of causal germline mutations (review in Bamshad et al. 2011) as well as for the discovery of somatic mutations involved in multiple diseases (Pagnamenta et al. 2012; Azizan et al. 2013; Yu et al. 2014; J. S. Lim et al. 2015).

Exome sequencing can be achieved by two different methods. The least common of them is amplicon-based exome sequencing, where primers complementary to the boundaries of exons amplify them specifically and the resulting product is sequenced. This method has higher false positive as well as false negative call rates and produces worse coverage uniformity (Samorodnitsky et al. 2015). The most common method is hybridization-based exome sequencing. RNA probes with exome sequences hybridize with the complementary DNA fragments, capturing them, while the rest is washed away. Then, the retained fragments are amplified and sequenced (R. Chen, Im, and Snyder 2015). Although this method is superior to amplicon-based methods, probes are synthesized with the reference sequence. Hence, if an individual is heterozygous for multiple close positions, the DNA fragments carrying the alternative allele will hybridize less effectively with the probes, reducing their capture efficiency. Fewer fragments with these variants will be present in the final sample so after sequencing, allele balance will be smaller than 50%, a phenomenon known as capture bias. Nonetheless, even if this is the main reason why exome variants are better found with whole genome sequencing, they only differ in about 3% of variants (Belkadi et al. 2015).

Several bases surrounding the exons are usually also captured, especially around short exons. Some of these bases are off-target, bases not intended to be captured but retrieved because fragments complementary to the probes contained them. Nonetheless, depending on the capture design, some intronic bases will be on-target, so their coverage is enough to discover variants. This allows to find intronic variants that can alter splicing donor or acceptor sites as well as positions involved in nonsense-mediated decay.

6.4 Other technologies useful for somatic variation analysis

Single cell sequencing

Unlike bulk sequencing, where multiple cells are homogenized into a single sample, techniques that allow the sequencing of individual cells have been recently developed (Marcy et al. 2007; Pushkarev, Neff, and Quake 2009). Since the amount of DNA present in an individual cell is insufficient for sequencing, whole genome amplification (WGA) needs to be performed first. Multiple methods exist to do this, such as multiple annealing and looping-based amplification cycles (MALBAC) (Elowitz et al. 2002) or more frequently used, multiple displacement amplification (MDA) (Dean et al. 2001). MDA amplifies a DNA sample by random priming using the bacteriophage Φ29 polymerase (Lázaro, Blanco, and Salas 1995), which amplifies continuous strands that are then displaced and again amplified. This produces a large amount of DNA from a very limited input sample. Unfortunately, biases are common, specifically, one of the DNA strands can be preferentially amplified to the point where allele dropout occurs. That is, one of the alleles is not amplified. Moreover, amplification can be uneven, producing a few very poorly amplified chromosomes (Borgström et al. 2017). Further, the type of lysis used in the protocol can influence the profile of errors obtained (Dong et al. 2017).

Single cell sequencing has been used to explore tumor heterogeneity (Gerlinger et al. 2012) and has also been proposed as a method for screening in-vitro fertilized embryos prior to implantation (Xu et al. 2016). Although it can be used to discover somatic variants, the cost of sampling enough cells to get a robust estimation of mutation frequencies in the tissue would be high. Nonetheless, it is very useful for validating somatic mutations found in bulk sequencing, especially systematic sequencing errors. They can be identified if a proportion of reads supporting a somatic mutation (1-30%) support the alternative allele in multiple single cells. Also, cell lineage trees can be inferred by using this technology (Frumkin et al. 2005).

Clonal expansion of single cells

A different strategy to amplify the genome of a single cell and to have enough material for sequencing is to culture it. Primary cultures can be derived by growing single cells from multiple tissues. This way, DNA is copied by replication and the repair machinery of the cell can ensure a high-fidelity process. Then, DNA can be extracted and sequenced from a bulk sample of the culture. Variants present in the original single cell will be inherited by all the culture cells and therefore will be in ~50% of the sequencing reads. On the contrary, mutations that appear during clonal expansion will be present in less cells and reads, and can be discarded subsequently (Alexej Abyzov et al. 2017). Nonetheless, certain variants could provide cells with proliferative advantage and as a consequence, be overrepresented in the culture. To ensure the somatic state of the variants, target sequencing or PCR of DNA obtained from a bulk sample of the same tissue from where the cells were obtained can confirm their presence even if their frequency is very low (Bae et al. 2018). Also, systematic errors would fit better with a clonal expansion variant and would be discarded.

Linked reads sequencing

Linked reads are those known to belong to the same DNA molecule. This is achieved by tagging high molecular weight (HMW) DNA molecules during library preparation. In the Chromium by 10x platform, about 10 long DNA strands are mixed with a barcoded gel bead and restriction enzymes in a microfluidics chip so that micelles are formed. Then, the micelles are incubated, and the DNA is partitioned and barcoded. The probability of two HMW molecules from the same locus to have the same tag is very low. This way, after sequencing the resulting reads, reads that map close to each other and share the barcode can be linked (Kitzman 2016). This knowledge can be used to resolve regions with lower complexity as well as to build haplotypes. In somatic mutation calling, linked reads can be very useful to determine heterozygous mutations, since even if the proportion of reads supporting one allele fits more with it being a somatic mutation, if each allele is phased with one of the germline haplotypes, it must be a heterozygous mutation. Also, artefacts can be detected if they phase with both haplotypes, because true somatic mutations only phase with one of the germline alleles, that of the strand where they first appeared. This methodology can be used both with bulk and single-cell sequencing strategies, including exome sequencing (Mortensen et al. 2019).

Third generation sequencing

New sequencing technologies have been established in the past few years. Instead of generating reads a few hundred base pairs long, third generation sequencing platforms produce single molecule reads as long as 100,000 bp.

Pacific Biosciences (PacBio) Single Molecule Real Time sequencing (SMRT) technology uses sequencing-by-synthesis and fluorescent emissions by the four differently tagged nucleotides incorporation but does so for long single molecules and without cluster amplification (Korlach et al. 2010). A more recent technology is the Oxford Nanopore MinION. DNA molecules pass through a nanopore and the disruption to the electric current is used to determine their sequence, without DNA synthesis (Stoddart et al. 2009). Although the potential utility of long reads for calling somatic mutations is obvious – they would help identifying heterozygous variants and artefacts from unresolved regions – higher and recurrent errors are still common (Carneiro et al. 2012; Laver et al. 2015), complicating their use.

7. Neurodegenerative diseases

Neurodegenerative diseases are those that result from the progressive loss of nervous system cells and functions. The greatest risk for such conditions is ageing. Thus, modern lifespan increase implies that the number of people suffering with neurodegeneration has and probably will increase. Alzheimer and Parkinson diseases are the two most common neurodegenerative disorders, whose causes are far from being elucidated.

7.1 Parkinson disease

Parkinson disease (PD) is the second most common neurodegenerative disorder. Though it is rare in the general population, prevalence increases with age, so that about 1% of people over 60 years of age and up to 4% in the highest age groups are afflicted (de Lau and Breteler 2006). PD cases have been reported all throughout history (García Ruiz 2004) but it was finally named after James Parkinson in 1817 for his dedication to the *shaking palsy*.

The most characteristic clinical manifestations of this progressive disease are resting tremor, bradykinesia, rigidity and postural instability (Hoehn and Yahr 1967). Its pathological features are the loss of dopaminergic neurons in the substantia nigra, a basal ganglia structure, and the appearance of Lewy bodies, abnormal accumulations of ubiquitinated proteins inside cells (Kuzuhara et al. 1988), which mainly contain α-synuclein, a presynaptic protein (Spillantini et al. 1997).

The substantia nigra has two main parts, pars reticulata and pars compacta. Neurons at the pars reticulata are GABAergic and thus inhibit multiple brain regions, modulating body and eye movement. On the other hand, pars compacta has mainly dopaminergic neurons which are involved in learning and reward-seeking through their connection to the striatum, the largest structure of the basal ganglia (Afifi 1994).

In 1960, it was discovered that PD patients presented an acute loss of dopamine in the caudate nucleus and the putamen (Ehringer and Hornykiewicz 1960), which led to the well-established therapy with levodopa, a drug by then already known to be decarboxylated to dopamine in the body (Holtz 1939). This loss is driven by neuronal death, which affects especially the ventral component of the substantia nigra (Bernheimer et al. 1973), a structure that by time of death has lost 50-70% of its neurons compared to unaffected individuals (Davie 2019). Pathological changes progress to other regions as the disease advances, and such progression is what determines the different Braak stages (Braak et al. 2003). Lewy neurites, fibrillar α-synuclein aggregates are more frequent in stage I, when structures of the lower brainstem and the olfactory nucleus are affected. Then, in stage II, they expand to the medulla oblongata. In stage III, Lewy bodies are more frequent, and they expand to the pars compacta of the substantia nigra. In stage IV, severe dopaminergic cell death in the pars compacta occurs. Also, the amygdala and thalamus are affected. In stage V, the disease expands to the neocortex and at stage VI motor and sensory areas in the brain are also affected (Braak et al. 2003; Jellinger 2009).

Some environmental causes for parkinsonism have been reported. Manganese intoxication induces parkinsonism without the appearance of Lewy bodies (Aschner et al. 2009) and because different structures are affected, such as the cerebellum (Perl and Olanow 2007), it cannot be considered as a cause of Parkinson disease, but just of parkinsonism. More importantly, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) causality of PD was accidentally discovered after heroin addiction sufferers consumed a synthetic opioid, desmethylprodine (MPPP) which was contaminated with MPTP (Lewin 1984; P. A. Ballard, Tetrud, and Langston 1985). MPTP is metabolized to 1-methyl-4-phenylpyridinium (MPP+), which has high affinity for the dopamine transporter dopaminergic neurons use to reuptake the neurotransmitter, thus making them more vulnerable to this toxin (Shen et al. 1985). MPTP has been used for inducing Parkinson in animals since. Interestingly, some herbicides such as Paraquat, have a similar chemical structure to MPTP, and it has been shown that continued exposure to them increases the risk of PD development (Koller et al. 1990; Van Maele-Fabry et al. 2012).

Since it is primarily a sporadic disease, a genetic cause was considered unlikely for a long time. However, in the 1990s some early-onset familial cases were reported (Duvoisin 1996) and finally, a dominant variant in the α-synuclein gene (*SNCA*) was identified as causal (Polymeropoulos et al. 1997). It was proposed that the missense mutation promoted self-aggregation by changing protein structure. Soon thereafter, α-synuclein was also detected in Lewy bodies of sporadic PD cases (Spillantini et al. 1997), and a second mutation in familial cases was discovered (Krüger et al. 1998) demonstrating the relevance of this protein in the development of Parkinson disease.

A different progression of the disease, juvenile parkinsonism, which has a very early onset (before the age of 40) and a very slow progression with no Lewy bodies or neurites at autopsy, has also provided insight into the molecular mechanisms by the identification of mutations in familial cases. This is how mutations in parkin, *PRKN*, were first described (Kitada et al. 1998). Parkin is a protein that attaches ubiquitin to proteins, tagging them for degradation, a process known as ubiquitination. Even more relevant loci have been discovered with family studies, such as *UCHL1* (Leroy et al. 1998), whose mutation creates a partial loss of its catalytic activity that results in aberrations in the proteolytic pathway and aggregation of proteins; once again highlighting the relevance of protein degradation pathways for the disease.

Among multiple other loci, microtubule associated protein tau (*MAPT*), though more famously associated with Alzheimer, has also been linked to PD by association within families (Martin et al. 2001). Pathogenic mutations in the *GBA* gene produce Gaucher disease – a lysosomal disease – in homozygosis, whereas in heterozygosis have been related to PD (Mata et al. 2008). Mitochondrial dysfunction has also been proposed to be involved in the development of the disease, with a hereditary form of Parkinson in consanguineous families caused by mutations of *PINK1* (Valente et al. 2004). Another monogenic form of parkinsonism is caused by mutations in *DJ1* (Bonifati et al. 2003) whose lack of function sensitizes cells to oxidative stress (Yokota et al. 2003). However, whether mitochondrial alterations result in malfunctions in the ubiquitin–proteasome system or insufficiency in protein degradation leads to mitochondrial damage is yet to be disentangled (Abou-Sleiman, Muqit, and Wood 2006).

Besides these monogenic familial cases, most PD cases are sporadic, and its estimated heritability is quite low, with 18% of concordance between monozygotic twins (Burn et al. 1992) when testing putamen dopamine uptake, and 25% of first-degree relatives of Parkinson patients having abnormally reduced putamen dopamine uptake (Piccini et al. 1997). A meta-analysis of genome-wide complex traits analysis (GCTA) also identified up to 27% phenotypic variance (Keller et al. 2012).

The link between PD and somatic mutations is unclear. A study on 511 sporadic cases did not find somatic variants in *SNCA* with a sensitivity limit at 5% of variant allele frequency (Proukakis et al. 2014). On the other hand, high levels of heteroplasmic mitochondrial DNA deletions in substantia nigra neurons were found (Bender et al. 2006) and neurons at the substantia nigra carried more SNCA gains than controls, positively correlating with age of onset (Mokretar et al. 2018).

7.2 Alzheimer disease

Alzheimer disease is the most common form of dementia, with millions of patients worldwide (Cornutiu 2015). It is characterized by impaired memory, judgment, decision making, orientation to physical surroundings, and language (Nussbaum and Ellis 2003). The pathological hallmarks are neuronal loss, extracellular senile plaques, and neurofibrillary tangles (Alzheimer 1907).

Similar to Parkinson disease, Alzheimer is a progressive disease. Hence, Braak stages are also used to characterize the advancement of the disease, determined by the appearance of neurofibrillary tangles in different brain regions independently of senile plaque progression. In stages I and II, neurofibrillary tangles are mainly confined to the entorhinal cortex. In stages III and IV limbic regions such as the hippocampus are also affected, and in stages V and VI there is extensive neocortical involvement (Braak et al. 2006). The entorhinal cortex is the first brain structure to be affected and its functions are memory and planning and spatial navigation, which some authors propose are two sides on the same coin (Buzsáki and Moser 2013).

Senile plaques are also referred to as amyloid plaques, a term coined by the German pathologist Rudolf Virchow in the XIX century because they appeared starch or cellulose-like. We now know they result from the polymerization of amyloid beta (Aβ) peptides. Aβ derives from the processing of the amyloid precursor protein (APP), which is concentrated at synapses. APP is cleaved by β-secretases and γ-secretases to form Aβ peptides. These peptides then form soluble oligomers which could be involved in the disease (Hsia et al. 1999; Shankar et al. 2008). When the oligomers polymerize into bigger structures, they form amyloid plaques (Alzheimer 1907).

Aβ was shown to be in senile plaques in both Alzheimer and Down syndrome patients (Masters et al. 1985). Although infrequent, duplications of *APP* cause autosomal dominant early-onset familial Alzheimer (Sleegers et al. 2006). Because they carry an extra copy of chromosome 21, where the *APP* gene resides, all Down syndrome patients that survive to age 40, something increasingly common, develop Alzheimer (Lott and Head 2005). Several mutations in *APP* were found in familial cases (Goate et al. 1991; Tanzi et al. 1987; Chartier-Harlin et al. 1991), which made Aβ polymerize at a higher rate. This led to the amyloid cascade hypothesis in the 90s (Hardy and Higgins 1992) which suggests that is the toxic effect of the plaques which results in synaptic alterations.

Neurofibrillary tangles are aggregates of hyperphosphorylated microtubule associated proteins tau (*MAPT*). The main function of this protein is the stabilization of microtubules, which is of especial relevance in axons and dendrites, where microtubules are fundamental to vesicle transport. When tau is hyperphosphorylated it dissociates from microtubules and polymerizes, forming the neurofibrillary tangles (Alonso, Grundke-Iqbal, and Iqbal 1996). Other diseases that have this same pathological element are called tautopathies (van Slegtenhorst, Lewis, and Hutton 2000).

Familial cases also identified other loci involved in Alzheimer disease. Presenilin 1, *PSEN1*, is a component of γ-secretase, one of the complexes that processes APP into Aβ (St George-Hyslop et al. 1987). Later on, *PSEN2*, also a component of the same complex was found (Schellenberg et al. 1992).

Altogether, heritability is estimated to be around 70% or higher than 90% for late and early onset Alzheimer disease, respectively (Ballard et al. 2011; Wingo et al. 2012). It is clearly a complex disease, where multiple markers explain a portion of the phenotypic variability (Ridge et al. 2013), nonetheless, some of it remains unexplained.

Finding the cause of the sporadic forms of neurodegenerative diseases supposes a great challenge. Since these cases are clinically undistinguishable from familial forms, somatic mutations have been proposed as a non-inherited genetic cause (Pamphlett 2004). Also, the fact that SNVs accumulate in neurons with age (Lodato et al. 2018) could explain late onset cases.

OBJECTIVES

1. Detect features affecting somatic variant calling

2. Explore the burden of exonic single nucleotide somatic variants in Parkinson disease

3. Assess the detection of somatic copy number variants in from aCGH data

4. Examine the abundance of somatic variants in a neurotypical individual in the context of the Brain Somatic Mosaicism Network

RESULTS

Somatic mutations in Parkinson disease patients

1. Data processing for somatic variant calling

To explore somatic mutations in Parkinson disease (PD), we sequenced the whole exome of five different tissues from ten patients. Blood was obtained from stored vials, while central nervous system samples — neocortex, cerebellum, substantia nigra and striatum — were collected during autopsies (table 1). Their exome was captured and sequenced.

Inspection of the resulting FASTQ files showed increased per base sequence (Fig. S1) and kmer content (Fig. S2) at the beginning and end of reads, that is, the same sequences appear frequently at read ends. This usually appears as a result of sequencing the ends of adapters ligated to each sample's DNA fragments for sequencing. Trimming algorithms can be used to remove these sequences, but always suppose a compromise between keeping adapter sequence and removing true sequence. This is because the algorithms look for portions of the known adapter sequence at read ends. If one chose to be very stringent, all reads starting with the last nucleotide of the adapter should be trimmed.
Further, the need to trim the reads disappears when using a mapper that performs soft-clipping, i.e., read starts or ends are masked when they do not align to the reference genome. Commonly used downstream tools take this masking into consideration, so that nucleotides coming from adapters will not be included when calling variants. This issue is especially relevant to consider when calling somatic mutations. Excessive trimming could imply losing a few reads supporting an allele, which can be crucial for variant calling. On the other hand, if reads are not trimmed, adapter sequences could be mistaken for somatic variation if not inspected carefully. Hence, a mapper that clips alignments — BWA — was used to map the untrimmed reads to the human reference genome.

The chosen reference was hs37d5, the version of the human genome used by the 1000 Genomes Project (Gibbs et al. 2015) Phase II. Coordinates match the standard hg19 reference, which enables the use of the more complete annotations and orthogonal information available for that reference. Additionally, hs37d5 contains known human genomic sequences: An Epstein-Barr virus (EBV) sequence and the decoy, which includes sequences coming from HuRef, BAC and fosmid clones and the *de novo* assembly of NA12878. In contrast, alternate loci are not included. The advantage the decoy provides is suggested by its name. Reads find an accurate alignment quicker, instead of spending time looking for more inexact matches. This improves mapping speed, and more importantly, reduces false positive variant calls.

This strategy resulted in high mapping percentages; between 99.92% and 99.98% of reads were mapped to hs37d5. FASTQ files were merged by sample, that is to say, each tissue of each individual. Duplications accounted for a median of 5.5% of reads. After their removal, secondary alignments were also excluded. This last step is important for minimizing unspecific mapping, which can generate false positives in somatic variant calling. After processing, the mean coverage on the target region was 60x per sample (table 1, Fig. S3). Relative mean coverage between the X and Y chromosomes was used to confirm each patient's reported sex (table 1).

Table 1. Sample information. Age at death, age at Parkinson onset, reported and genomic (Y/X) sex for each individual. Mean coverage over the exome per tissue and patient is indicated.

				Mean coverage					
Patient ID	**Sex**	**Age**	**Onset**	**Blood**	**Cerebellum**	**Striatum**	**Neocortex**	**Subst. nigra**	**Y/X**
DV1	Female	87	NA	57	58	57	57	57	0
DV2	Male	83	51	60	59	47	60	63	1
DV3	Female	81	68	59	61	60	61	63	0
DV4	Female	96	69	60	60	59	66	61	0
DV5	Female	81	NA	65	64	60	67	65	0
DV6	Male	76	51	61	61	61	64	61	1
DV7	Female	82	54	60	61	61	61	59	0
DV8	Male	79	NA	59	57	58	58	58	1
DV9	Male	50	27	61	57	57	58	56	1
DV10	Female	78	56	57	57	50	58	60	0

2. Germline variants

HaplotypeCaller with standard parameters was used to call germline variants for each sample. Principal components analysis (PCA) of the obtained SNPs suggested that the cerebellum sample from individual DV2 (DV2C) could instead belong to DV8 (Fig. 19). In addition, compared to the other individuals, DV2's blood sample was distant to the rest of DV2's tissues. Manual inspection of a few variants confirmed this, showing DV2C shared variants with DV8 and not with the other DV2 samples while DV2B had a smaller allele fraction of those same variants, suggesting a mixture of genomes in that sample (Figs. S4 and S5). Because of this, DV2 was excluded from somatic variant calling. Still, its samples were considered for noise profiling.

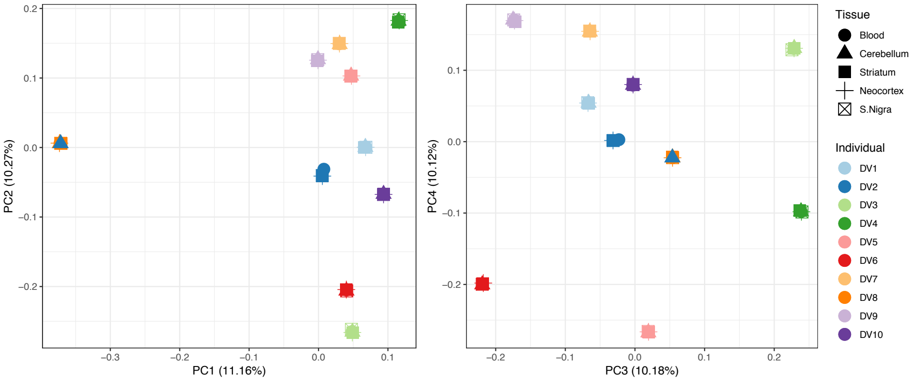


Figure 19. Germline variants PCA. Plots showing the four first principal components with the amount of variance explained by each of them in parenthesis.

2.1. Germline variants in known PD genes

We first checked germline variants in genes previously related to PD and reported at OMIM (table S1). Combined Annotation Dependent Depletion (CADD) score integrates multiple annotations into a single metric by contrasting simulated variants to those that were not removed by natural selection. Specifically, a CADD > 15 (Rentzsch et al. 2019) was used to select putative deleterious mutations. Two variants, rs17651549 and rs12595158, had high values (table 2), indicating that they are amongst the 0.6% most deleterious mutations that can occur in the human genome. Variant rs17651549 is a missense mutation of the gene MAPT predicted as deleterious by multiple methods (table 2) and at a position highly conserved in vertebrates. The specific variant has been previously linked to PD by different means: multivariate family-based association tests (K. S. Wang, Mullersman, and Liu 2010), pathway analysis (Song and Lee 2013) and targeted resequencing (Spataro et al. 2015). However, a contradictory haplotype association analysis has shown it to provide a reduced risk for PD (J. Li et al. 2018). It is not infrequent in Europe; particularly, the frequency of the heterozygous genotype (C|T) in the 1000G IBS population (Iberian populations in Spain) is 0.383. Among our reduced number of individuals, the frequency is higher, 6 out of the 10 are heterozygous.

Another missense variant, rs12595158, overlapping the gene *VPS13C*, was heterozygous in one of the individuals, DV3. *VPS13C* is involved in lipid dynamics and has been shown to tether the endoplasmic reticulum to endosomes and lysosomes (Lesage et al. 2016). Although rs12595158 is predicted as deleterious by multiple methods and variants in the *VPS13C* gene have been linked to the early onset autosomal-recessive form of PD (Lesage et al. 2016; Schormair et al. 2018), and to PD in general via GWAS (Safaralizadeh et al. 2016), this specific variant has not been associated to any disease. The frequency of the C|T genotype in IBS is 0 (0.054 pulling all European samples) but it is more frequent in South Asian, East Asian and especially America, with more than half of Peruvians in Lima (PEL) being heterozygote. Its relevance could depend on the genomic background of different populations.

Table 2. Germline variants related to Parkinson disease. Variants identified both by genotyping PD related genes at OMIM and single nucleotide polymorphisms (SNPs) previously associated to the disease by GWAS studies. 1000 Genomes Project populations with the highest frequency are in parenthesis (IBS: Iberian populations in Spain, TSI: Toscani in Italia, PEL: Peruvians from Lima, Peru, GIH: Gujarati Indian from Houston, Texas, MSL: Mende in Sierra Leone, CHB: Han Chinese in Beijing, China). Effect predictions are encoded as deleterious (D), possibly damaging (P), tolerable (T), benign (B) and neutral (N).

Strategy	PD genes	PD genes	GWAS SNPs	GWAS SNPs	GWAS SNPs	GWAS SNPs
dbSNP ID	rs17651549	rs12595158	rs34884217	rs1801582	rs7412	rs2010795
hg19 coordinates	17:44061278	15:62316035	4:944210	6:161807855	19:45412079	21:45172628
Ref	C	C	A	C	C	G
Alt	T	T	C	G	T	A
Gene name	*MAPT*	*VPS13C*	*TMEM175*	*PARK2*	*APOE*	*PDXK*
Type	Missense	Missense	Splice acceptor	Missense	Missense	Intronic
Change	R370W	R153H	-	V352L	R202C	-
Alt frequency 1000G EUR	0.231	0.034	0.083	0.150	0.066	0.337
Alt frequency 1000G IBS	0.266	0	0.154	0.229	0.056	0.369
Highest population alt frequency	0.36 (TSI)	0.43 (PEL)	0.154 (IBS)	0.301 (GIH)	0.141 (MSL)	0.510 (CHB)
CADD phred	25.2	22.5	14.02	10.30	26.3	5.656
GERP	5.51	4.98	4.9	4.71	5.09	-2.97
SIFT	D	D	T	T	D	NA
Polyphen2 HDIV	D	D	B	B	D	NA
Polyphen2 HVAR	P	P	B	B	D	NA
MutationTaster	P	P	D	P	D	NA
PROVEAN	D	N	D	N	D	NA
phastCons100way Vertebrates	1	1	1	0	0.986	0
Individuals	DV3, DV4, DV5, DV6, DV8	DV3	DV3, DV4	DV1, DV3, DV4, DV6, DV8, DV9	DV5, DV6, DV10	DV1, DV3, DV4, DV6, DV8, DV9, DV10

2.2. Polymorphisms previously linked to PD

Then, we interrogated other polymorphisms previously linked to PD by GWAS (table S2) (E.-K. Tan et al. 2010; Do et al. 2011; International Parkinson Disease Genomics Consortium 2011; International Parkinson Disease Genomics Consortium and Wellcome Trust Case Control Consortium 2 2011; Lill et al. 2012; Nalls et al. 2014a; C.-M. Chen et al. 2016). Even if they overlap genes linked to PD, our previous analysis was not able to pick them up because their CADD score is not high enough (table 2). However, we can make use of the power that large GWAS studies have to pinpoint interesting variants. Four of the linked polymorphisms were heterozygous in at least one of our individuals:

DV3 and DV4 were heterozygous for a splice acceptor variant at *TMEM175*, rs34884217. This variant has been associated to PD (Nalls et al. 2014b; Heckman et al. 2017) and affects a gene in a site predicted to affect nonsense-mediated decay on a gene whose deficiency has been linked to the increase of α-synuclein aggregation (Jinn et al. 2017), indicating a possible causal link.

A heterozygote variant found in six of our individuals, rs1801582, is a missense mutation of *PARK2*. It is not well conserved in vertebrates and its frequency in IBS is 0.421. However, a meta-analysis reported an odds ratio (OR) of 1.36 in Caucasians when considering a heterozygote model (Ramakrishnan et al. 2016), making it an interesting variant.

Three individuals (DV5, DV6 and DV10) carried the alternative allele at rs7412. It was picked up because it is a missense variant at the *APOE* gene predicted as deleterious by most methods (table 2). Together with another nonsynonymous mutation, rs429358, it determines *APOE* allele. The reference version, TC (at rs429358 and rs7412, respectively) is known as *ApoE-ε3*. Most of the individuals are homozygous for this version. Three individuals carry *ApoE-ε2* (TT) in one of their alleles. While *ApoE-ε4* has been linked to increased risk of Alzheimer disease (Saunders et al. 1993; Michaelson 2014), *ApoE-ε2* has been modestly linked to increased risk of PD (X. Huang, Chen, and Poole 2004; Williams-Gray et al. 2009) although a bigger study showed no association (Federoff et al. 2012).

Finally, rs2010795, an intronic position on the pyridoxal kinase gene (*PDXK*) was variable among our individuals, with 4 being heterozygotes (DV1, DV6, DV8, DV9) and 3 homozygotes (DV10, DV3, DV4). Frequencies of those genotypes in IBS are 0.495 and 0.121, respectively. Interestingly, *PDXK* was identified as differentially expressed in dopaminergic neurons of PD patients and controls. The rs2010795 polymorphism was subsequently found to be associated to PD in German, British and Italian cohorts (Elstner et al. 2009) although a bigger sample of Italians revealed no association (Guella et al. 2010).

2.3 Deleterious variants in the exome

In addition, all germline SNVs obtained were annotated to look for any damaging mutations even if not previously linked to PD. Limiting variants to those with CADD > 15, predicted by SIFT as deleterious and with a frequency in the 1000G European population < 0.1 resulted in a total of 241 variant positions. Overrepresentation enrichment analysis (ORA) (B. Zhang, Kirov, and Snoddy 2005) for molecular function with all protein coding genes as background showed significant enrichment for "kinesin binding" (Fig. 20), driven by proteins involved in axonal transport that have been previously associated to PD: *CLSTN1* (Chuang et al. 2017; Kong et al. 2018), *KIF1B* (J.-M. Kim et al. 2006; Kedmi et al. 2011) and *KTN1* (van Dijk et al. 2012); to Alzheimer: *KCNC1* (Boda et al. 2012) or to amyotrophic lateral sclerosis (ALS): *TTBK2* (Liachko et al. 2014). Other significantly enriched molecular functions included some genes that could be related to PD phenotype, such as *ATP13A5* (Sørensen et al. 2018).
Contrarily, disease ORA did not result in any significant result.

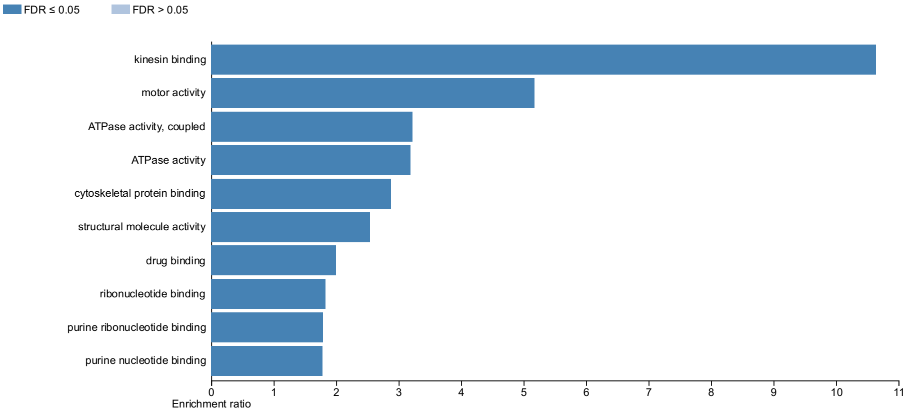


Figure 20. Germline variants enrichment analysis. Overrepresentation enrichment analysis for molecular function of germline variants predicted as deleterious and with low frequency in the Spanish population.

3. Somatic variants

Standard variant callers, such as HaplotypeCaller, are designed to find germline variants. They assume the potential genotypes in a diploid sample are homozygous for the reference allele (0/0), heterozygous (0/1) or homozygous for the alternative allele (1/1). Since they try to fit allele frequencies to this expectation, they are unsuited for somatic variant calling. While higher frequency somatic variants may be called as heterozygous positions, low frequency somatic variants will be classified as homozygous reference positions, i.e., invariant, interpreting alternative allele support as noise. Thus, calling somatic variants requires a more sensitive approach.

Somatic variant callers' development has been focused on cancer. Theoretically, cancer causing mutations should be present in the tumor but absent in the surrounding healthy tissue. As a consequence, many callers require matched "tumor" and "control" samples, so that they can identify the variants exclusive to the tumor. However, recent research has shown that somatic mutations are present in multiple tissues, even if they come from different germ layers (Lodato et al. 2015). Although lower frequency mutations or those whose high frequencies result from clonal expansion can be tissue exclusive, these are out of the scope of this study, due to the limited power provided by our sequencing coverage. With a mean of 60x per tissue and requiring a minimum of 3 reads supporting the alternative allele, we would call variants with a frequency of 5% or higher, which we expect to be in other tissues (Lodato et al. 2015). However, blood may have some exclusive somatic variants because of clonal expansion.

Nevertheless, our statistical power is increased by having five different tissues from each individual. Even if variants have a frequency below noise levels, in general, noise is quite random, so the presence of a variant in multiple tissue samples would strongly suggest the existence of a true somatic variant.

3.1. Exploring the biases affecting somatic variant calling

We tested two different approaches to call somatic single nucleotide variants (SNVs) that do not require matched samples. The first of them was a lax VarScan 2 calling (Koboldt et al. 2012). The goal was to get every variant position that passed a minimum base quality threshold and then explore the importance of different features of the data in predicting the quality of the call.

Because callers return variable positions, an absence of call could be due to either absence of alternative allele support or absence of coverage at that position. In order to be able to differentiate between these two scenarios, the five tissue samples of each individual were genotyped together.

Number of reads supporting the alternative allele

Standard VarScan 2 mpileup2snp parameters fix a harsh frequency threshold at 20%. This, together with other parameters, limits the discovery of somatic variants. In order to get every variant position, we used *--min-coverage 1 --min-reads2 1 --p-value 1 --min-var-freq 0.000001 --output-vcf*. This configuration only limits calling to the variants reported by samtools *mpileup*. For this reason, the raw output from VarScan 2 contains mostly sequencing errors, resulting in almost 7 M positions reported per individual. Requiring at least two reads supporting the alternative allele along the tissues of an individual drastically reduced the number to around 0.6 M. This is expected because virtually all positions with just one read supporting the alternative allele along an individual's tissues are due to random sequencing noise, so the probability of this happening is equal to the random Illumina sequencing error. However, the probability of getting two errors at the same position is much lower, it is two times the random error multiplied by the error specific to that type of substitution. This makes random errors decrease quickly when requiring higher numbers of reads supporting an allele. Nonetheless, random sequencing errors are still considerably more frequent than true events, as evidenced by the number of variant positions retained after this filter, so other features have to be considered to discriminate them.

Up to this point, filtered calls were too numerous to be true somatic variants, so a random set was inspected manually in IGV (J. T. Robinson et al. 2011) in order to pinpoint the most frequent confounding factor. Once the biggest source of false positives was addressed, the resulting calls were inspected again in search of additional biases. This recurrent procedure determined multiple features that help to identify true somatic variants, which we describe next, in a logical rather than a chronological order.

Multiple alleles called at the same position

Multiallelic calls tend to be noisier, because the probability of getting different substitution type errors at the same position is much higher than that of getting the same type of substitution. This has been recently reported (J. Kim et al. 2019). Excluding them from further analyses reduced calls in half.

Biased sequencing errors

Non-random errors are caused by library preparation damage, such as G>T substitutions caused by oxidation (Costello et al. 2013), and amplification error, which gets higher the more PCR cycles are performed (Brodin et al. 2013). Some positions in the genome are more prone to damage or harder to copy for polymerases, so recurrent noise occurs. Besides, not every Illumina sequencing error is random; the sequencing of certain positions always results in a small proportion of reads supporting an incorrect allele (Meacham et al. 2011). These systematic or biased errors are very difficult to distinguish from true somatic variants individually and represent a big obstacle for correct somatic variant identification. Since they are recurrent, having a panel of control samples greatly facilitates their identification, because variants found in several individuals in a somatic frequency are very unlikely to be true somatic variants. The influence of biased errors is illustrated by Fig 3. Positions whose total alternative depth (AD) is 2 along the five tissues are mostly private to the individual in which they were called. This shows these are random sequencing errors, not probable to be shared by multiple individuals. Increasing total AD decreases the proportion of unique calls such that, by total AD of 5, most positions are called in other individuals, indicating this part of the distribution is dominated by recurrent sequencing errors. At total AD ~50, which with a mean coverage of 60x per tissue corresponds to a frequency of 15%, variants are more individual-specific. Germline heterozygous positions (AD ~ 150) are, as expected, shared by higher numbers of individuals. A similar pattern is observed for particularity of calls by variant allele frequency (VAF) (Fig. S6). To consider this when filtering calls, we annotated the number of samples with support for the same alternative allele and then required a minimum of 2 AD, to get a better picture of biased errors.

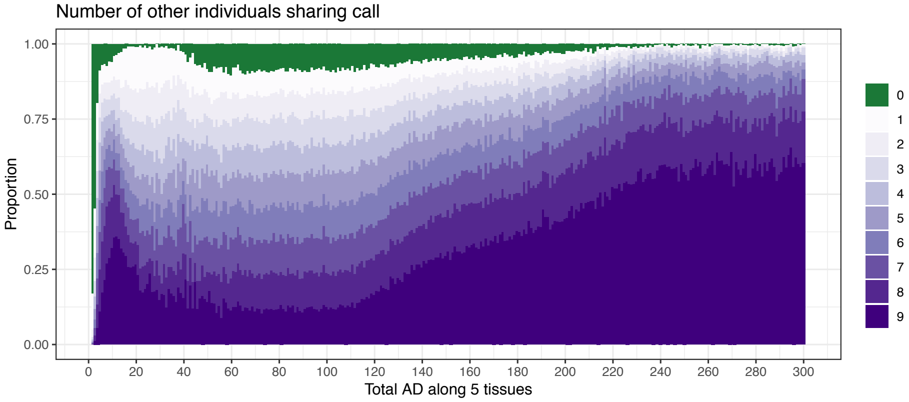


Figure 21. Singleness of calls by total alternative depth. Each bar shows the proportion of calls with a given alternative depth along the five tissues of an individual that have been called in none (0), or 1-9 other individuals. Calls were filtered so that their total depth was between 250 and 350.

R1 vs R2 bias

In Illumina paired-end sequencing, adaptors are directional. This allows us to distinguish reads sequenced in the original strand orientation, which are known as read 1 (R1) from those sequenced in the reverse complement orientation, read 2 (R2). When library preparation produces the oxidation of Gs in the original strands, they are incorrectly paired to As in the first PCR cycle and thus changed to Ts in the second PCR cycle. This creates an imbalance where R1 carries G>T changes while R2 carries the reverse complement, C>A changes. On the contrary, true variants would produce the two types of changes in both R1 and R2 (L. Chen et al. 2017). The R1/R2 ratio is more dispersed than a binomial distribution (Fig. S7), indicating more than sampling error is causing them. Although read pair differences are not as symmetrical in reality as the model suggested, variants with highly imbalanced R1 to R2 ratios are likely to be spurious. Further, because our coverage implies just a few reads would support a somatic variant, the power of any statistical test to identify this bias in a single tissue would be very limited. Still, callers do not annotate information on R1 and R2 read counts, much less stratified by allele. In order to be able to consider this information for our final calls, we developed a custom python script to annotate read pair allele counts (RAC) to VCFs.

Somatic “non-callable”

In addition to experiment-specific noise, there are regions of the genome less accessible to next generation sequencing technologies, especially when using short reads. Calling variants in these regions is especially challenging, and more so for somatic variants.

The 1000 Genomes Project generated a mask accumulating information from the numerous samples they sequenced. It reports positions where depth of coverage is much higher (H) or lower (L) than average, where many reads have a mapping quality of zero (Z), where the average mapping quality is low (Q) or positions where no reads align (0). Specifically, the strict version of the 1000G mask requires that total coverage is within 50% of the average, that no more than 0.1% of reads have mapping quality of zero, and that the average mapping quality for the position is 56 or greater. It overlaps with 23.1% of the non-N bases of the genome, but since it singles out its most unique regions, and the exome is enriched in such sequences, it only overlaps 7% of our target region. However, a bigger proportion of raw on-target calls fall within those regions (Fig. 22), proving the 1000G mask is able to identify noisier regions.

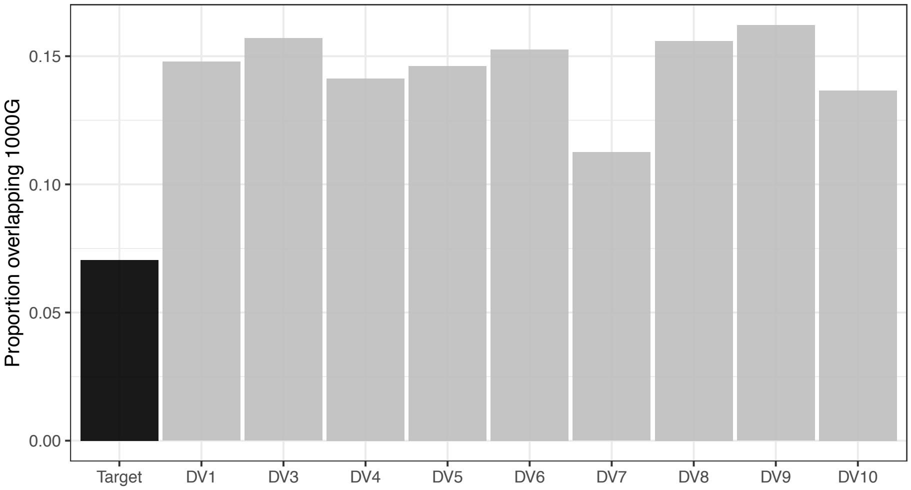


Figure 22. The 1000G strict mask overlaps with on-target VarScan 2 calls. Bars show the proportion of the target region (black) and raw on-target calls per individual (grey) overlapping the 1000G strict mask.

Adding to short read accessibility, mappability is the score that indicates the uniqueness of a genomic region given a read length. It is calculated by breaking the reference genome into read-length kmers and mapping them against the same reference genome. Regions where a single kmer aligns can be uniquely mapped with the given read length and their mappability is defined as 1. Regions where multiple kmers align are not uniquely mappable and have lower mappabilities. Since inaccurate mapping can produce false positives, we masked regions with mappability lower than 1 for our read length, 100bp. This only represents 0.35% of the exome and ~80% of it overlaps the 1000G strict mask (Fig. 23).

Finally, segmental duplications are an important source of false positive somatic variant calls. This is because different copies frequently carry different variants, so aligning the reads belonging to one of the copies to the other generates artefacts that are otherwise difficult to distinguish from true positives. Just over 45% of this track overlaps with the 1000G strict mask (Fig. 23), evidencing multiple segmental duplication regions are H or Q bases.

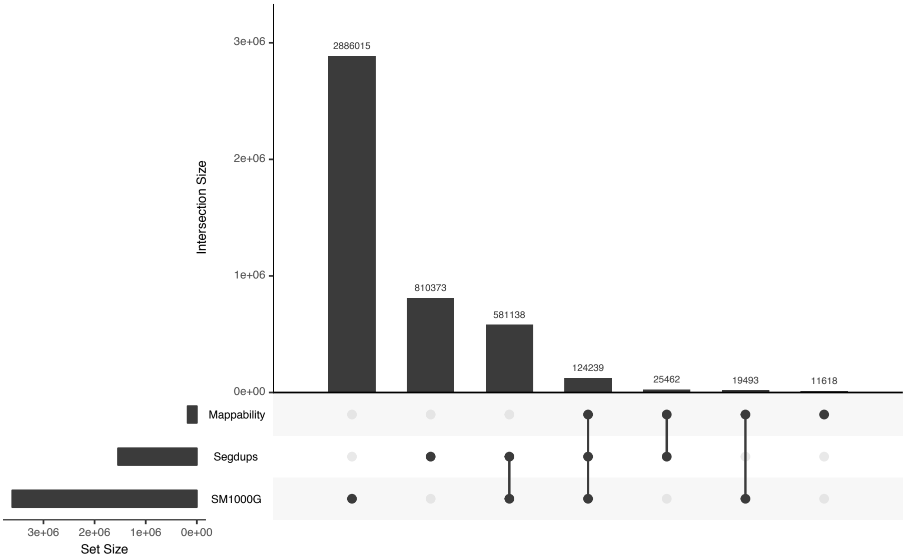


Figure 23. Overlaps between masking tracks over the target region. Horizontal bars show the number of target region base pairs overlapped by each of the three masks: 1000G strict mask (SM1000G), mappability for 100-mers (Mappability) and WGAC segmental duplications track (Segdups). Vertical bars indicate the number of positions in each intersection. Masks involved in each intersection are indicated by dots.

In summary, regions overlapping the 1000G strict mask, with mappability smaller than 1 or overlapping WGAC segmental duplications track constitute the portion of the genome we consider non-callable for somatic mutations. It supposes 9.8% of the target region or a little over 5Mbp in total. We annotated them in the VCFs so that we could remove them.

Copy number variants (CNVs)

Once the somatic callable positions in the genome have been singled out, there are still multiple confounding factors one has to take into account. One of the most obvious sources of false positives are copy number variants (CNVs). With a similar logic to segmental duplications, when an individual has a germline CNV, it is likely that the non-reference copy carries single nucleotide variants within it. If the reference genome has only one copy included, reads coming from all copies will be mapped to it, collapsing them into one locus. If there are only two copies, because the non-reference copy has different boundaries, its mapping rate will be lower, reducing the frequency of its variants to a frequency rather smaller than 50%, making them look like somatic variants. When a CNV consists of more than two copies, copy-specific SNVs will invariably look like somatic variants. This can be tackled more clearly by, depth of coverage. Collapsed reads coming from both copies significantly increase depth at the region. An example can be seen in Fig. 24: All DV1 tissues have a T>C at 1:145115820 with a VAF between 17 and 23%, which would make it a reasonable candidate somatic SNV. However, the same variant is found in other individuals such as DV10, whose tissues have similar frequencies (19-27%) at that position. Depth at the region is over 170x for both individuals, almost 3 times the mean coverage, pointing towards a common CNV with at least 3 copies being collapsed in that region.

Depth of coverage is typically variable along the genome and more so along the exome after its capture. For this reason, calling CNVs can be challenging in exome sequencing data. Also, CNV callers, as any calling algorithm, try to limit false positive calls, so they only return the most reliable instances. However, we should consider any possible copy number variable region because germline CNVs are more frequent than detectable somatic SNVs (Campbell and Eichler 2013; Bae et al. 2018). To address this, we first called CNVs with XHMM (Fromer et al. 2012a) and annotated calls in the VCFs and secondly, we required candidate positions to have a total depth (DP) between 20 and 100, which removes the more extreme 25% of the depth distribution.

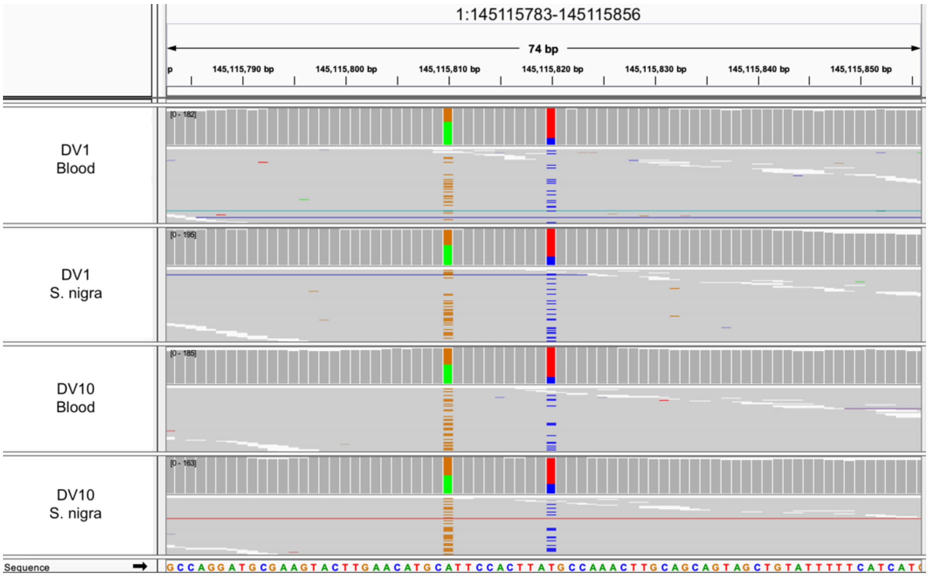


Figure 24. Example of population CNV variant creating a FP somatic mutation. A T>C change is seen in ~20% of DV1's blood and substantia nigra reads. Coverage in the region is almost 3 times the mean, >170x. DV10's tissues show a very similar coverage and C frequency.

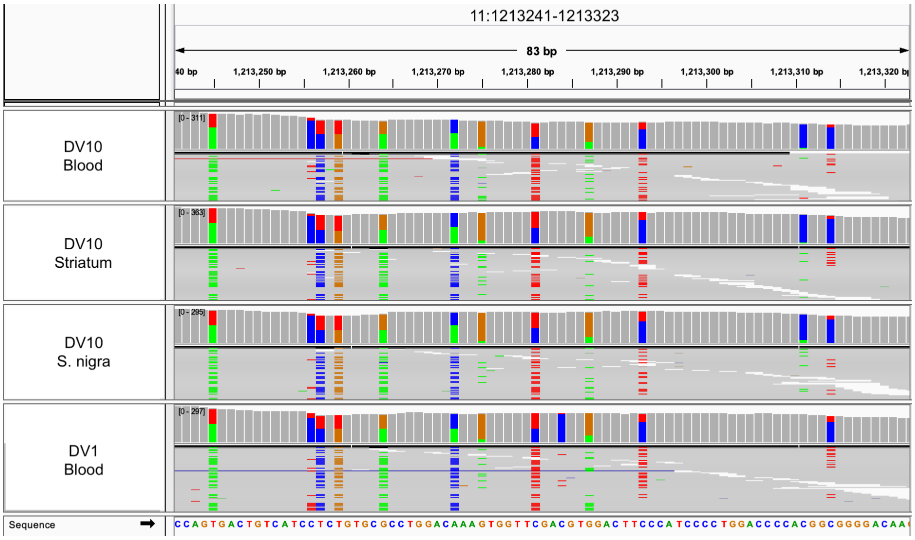


Figure 25. Example of clustered variants as a proxy of CNV presence. Region with coverage >300x and multiple variants in 10-20% frequencies in multiple tissues of DV10. Many of them are also observed in DV1.

Additionally, at times, copies present multiple contiguous variants, which look like clustered somatic variants when collapsed (Fig. 25). Because localized somatic hypermutation — kataegis — is not expected in non-cancer tissues, we only considered a candidate if at most three other variants were within read-length distance. This is demonstrated when looking at the other filters targeting variants removed by this filter (Fig. S8).

Biased position in reads

VarScan 2 called some variants with an expected somatic VAF in multiple individuals, suggesting those variants were actually artefacts. Taking a closer look, we observed that reads supporting the alternative allele carried it in the same portion of the read and they only aligned to a certain fraction of the region (Fig. 26). Further, reads supporting the alternative alleles were clipped at both ends, indicating that sequence at the ends of the reads does not align to the genomic region. In contrast, reference allele position in the reads was unbiased, i.e., there were reads carrying it in different portions of the reads and reads aligned to the surrounding regions. These regions are not included in the 1000G strict mask nor in the segmental duplications track and they were not excluded by our 100-mers mappability track or when removing secondary alignments and at the same time are present in many individuals. Because of these reasons, they most probably come from unresolved regions in the genome. These must be parts of the genome that share a portion of their sequence with a known region but are not included in the genome. This makes reads coming from the unresolved region map to the known region by clipping the parts of reads that overlap the sequences private to the unresolved region, while differences in the shared sequence will look like variants. Because the mapping efficiency is extremely limited by the non-homologous portions surrounding the common sequence, variant allele frequency is reduced, making them look like somatic mutations without increasing coverage significantly. To address this, we developed a score, PIR (position in reads) which indicates a bias of an allele towards the first, middle or last third of the aligned reads (PIR=1 2 or 3 respectively) when more than 90% of the reads carry the allele in that portion; or towards none (PIR=4) if that is not the case (Fig. 27) and annotated it for each allele at each call with our custom python script.

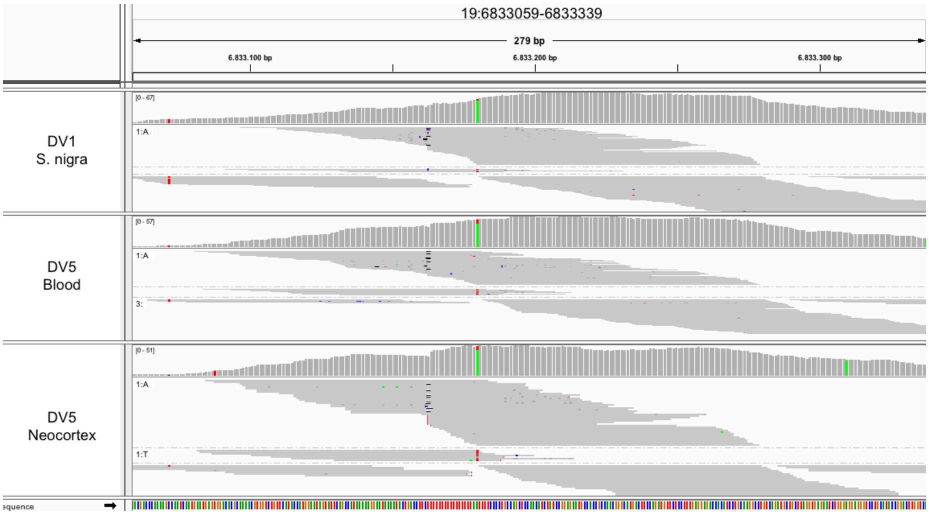


Figure 26. Biased position in reads example. Reads are ordered by their allele at 19:6833180.

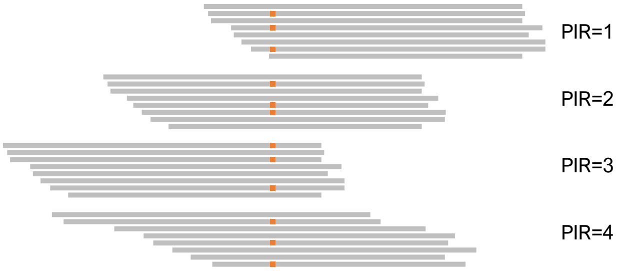


Figure 27. Position in reads or PIR score. Grey lines represent reads and the orange square a mismatch to the reference. When variants are concentrated in one of the thirds of the reads, PIR score is 1, 2 or 3. If they are carried by reads without a position bias, PIR score value is 4.

Strand bias

When calling germline SNVs, calls whose strand bias falls in the most extreme 10% are commonly excluded (Guo et al. 2012). However, subtler strand imbalances can affect one allele more than the other, lowering its frequency and making it difficult to distinguish from a somatic SNV. The example in Fig. 28 shows a T>C variant exclusive to DV4 where most of the alternative allele supporting reads are R1 (pink) reads. This phenomenon can be caused by sampling errors when sequencing, especially with lower coverages, but it can also arise when processing data by applying local realignment and base quality score recalibration (Guo et al. 2012). We used a Poisson test for evaluating the overall strand imbalance and a Fisher exact test (FET) with strand counts stratified by allele to find cases where strand bias affects only one allele and we annotated on the VCFs with our custom python script. However, true low frequency somatic SNVs are supported by very few reads at each tissue, so filtering by FET p-value can be too stringent.

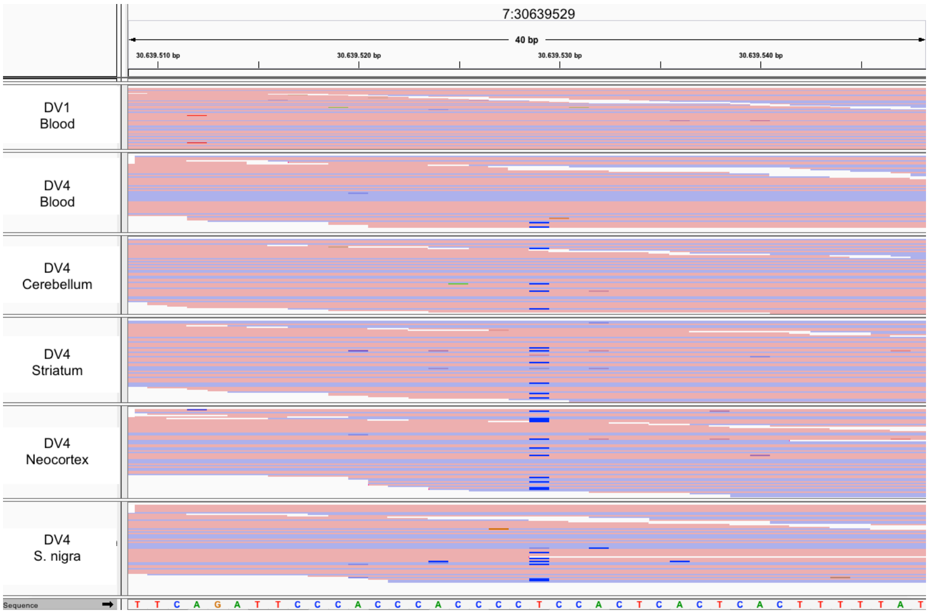


Figure 28. Example of strand bias. Region with adequate coverage per sample (~50x). Reads are colored by strand: plus or forward (pink) and minus or reverse (blue). Most reads supporting the alternative allele are forward reads.

Indels and homopolymers

Aligning reads to regions of the genome with small insertions or deletions (indels) or with homopolymers is especially challenging. This is why best practices pipelines include local realignment steps. Still, these regions are a source of false positive somatic calls, because a small proportion of reads mapped incorrectly can place allele support where it does not belong. To address this, we excluded variants found within 5 bp of an indel. An example of a false positive variant within an indel can be seen in Fig. 29. Tissues from multiple individuals carry the indel, probably in a heterozygous state. A small proportion of reads carry an A>C variant, but because it is in multiple individuals, it is an artefactual call.

Also, we observed several cases of calls found adjacent to homopolymers, and whose alternative allele was the homopolymers' nucleotide. Some calls, such as the G>A in Fig. 30 are within stretches of a nucleotide, which makes polymerase replication errors very probable. Allele support for A can be seen in all the Gs in the region in different individuals. For this reason, we did not consider variants within this scenario.

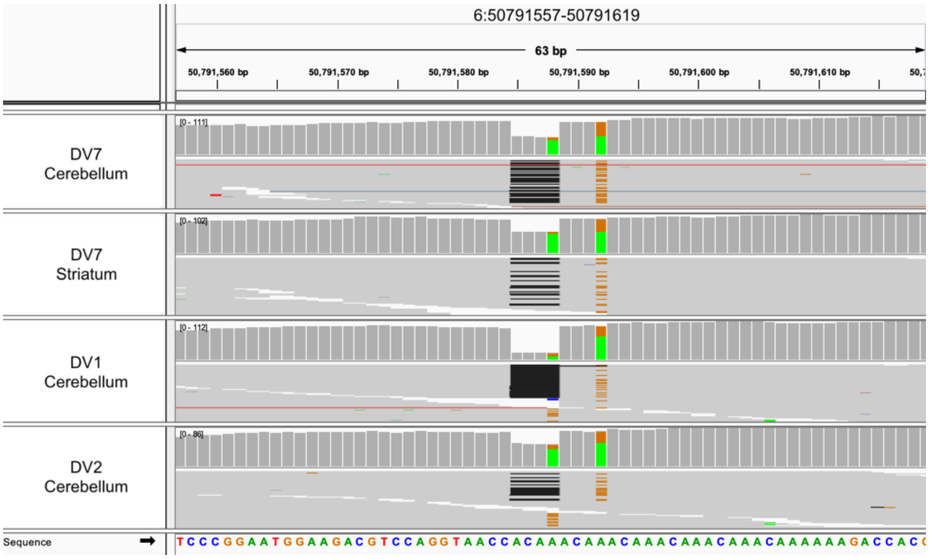


Figure 29. Example of artefactual call around an indel. Black horizontal lines represent deletions in the reads. Multiple individuals carrying the indel in a heterozygous state have a few reads supporting an A>G change.

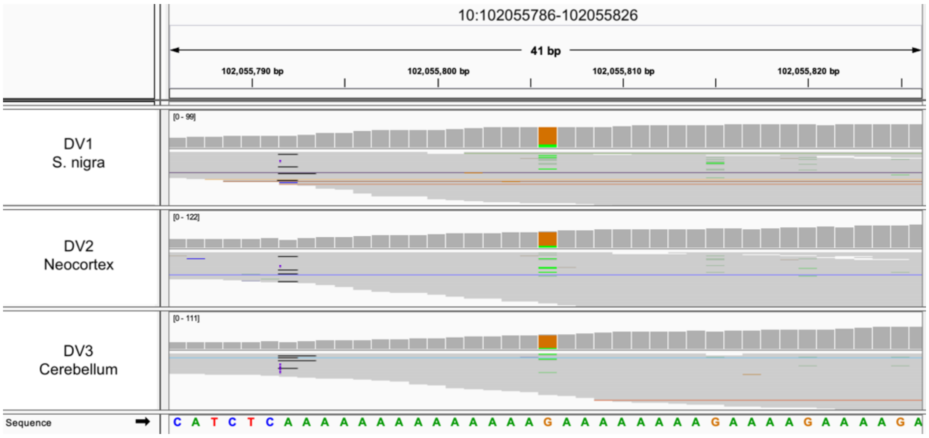


Figure 30. Example of errors at a nucleotide immersed in a homopolymer. In a region with A homopolymer stretches, 10-15% of bases aligned to a G carry an A allele in multiple samples. The same can be observed at positions 102055815, 102055820 and 102055825, other Gs surrounded by As.

Heterozygosity

One could expect variant allele frequency (VAF) to be crucial for differentiating somatic from heterozygous variants. The latter should have VAFs close to 50% while somatic variants should have a smaller frequency. Yet, we found allele balance dispersion in high-confidence heterozygous positions to be higher than that of a binomial distribution (Fig. 31). High-confidence heterozygous positions are those that are included in the common variant dbSNP database, are called in all tissues of one of the individuals (excluding DV2) and have a VAF between 0.45 and 0.55 and a depth from 20 to 100 in at least one of the tissues. Although allele balance is shifted in favor of the reference allele (reference bias), higher VAFs are also more frequent than expected (right part of the distribution). As the distribution gets narrower with higher sequencing depth (Fig. 32), we can infer a significant portion of the error comes from allele sampling.

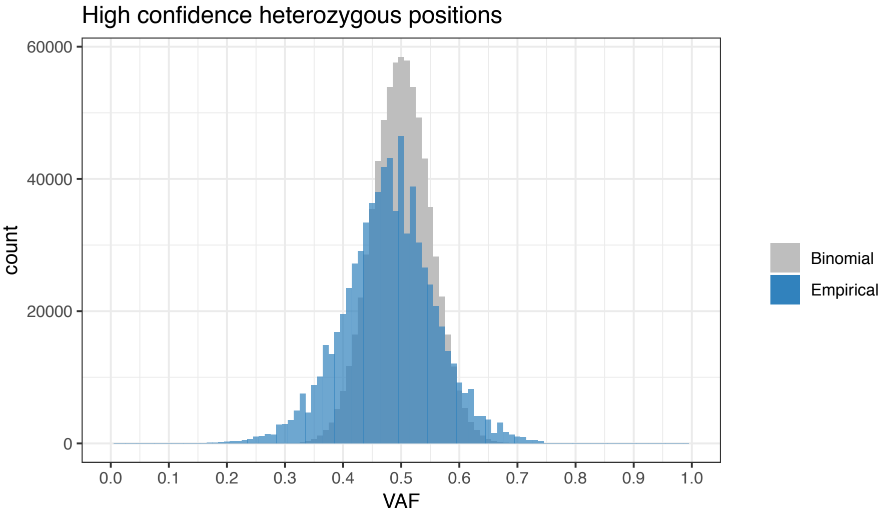


Figure 31. Variant allele frequencies at heterozygous positions. Histogram of VAFs at high-confidence heterozygous positions (blue) vs a random binomial distribution with p=0.5 (grey). High-confidence heterozygous positions are those present in the common dbSNP database, where all tissues of an individual are called as variant and at least one tissue has a VAF between 0.45 and 0.55 and a depth of 20 to 100.

This dispersion makes the heterozygous VAF distribution overlap with the theoretical somatic range, such that a single position's VAF does not give much information. Having five tissues from the same individual greatly improves our power to determine heterozygous positions (Fig. 33). While 18.4% of high confidence heterozygous positions have a significant binomial test (p-value <0.05), meaning that they would not be considered as heterozygous variants, including the information of one other tissue reduces the proportion to 3.9% if we consider one non-significant test as proof of the position being heterozygous. If we include 4 tissues, we can get as few as 0.16% (SD=0.019) misclassified, so the binomial test p-value for VAF was annotated for each sample and call with our custom python script. Also, in order to add more information to help us differentiate heterozygous variants, we annotate variants present in the common variant dbSNP database, those present in >1% of the population.

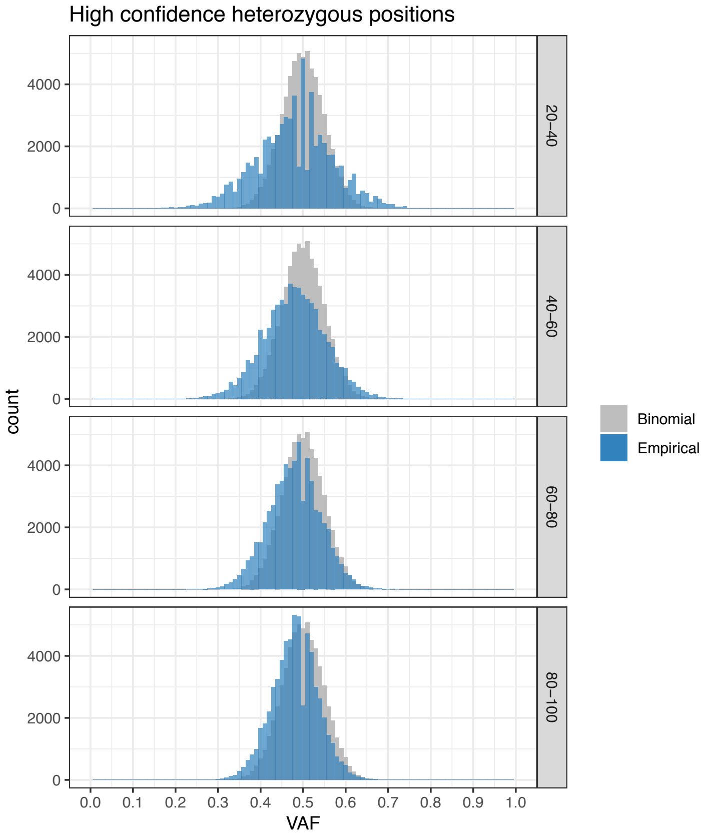


Figure 32. Variant allele frequency dispersion gets smaller at higher depths. VAF was stratified by coverage window (Y axis facets). Random samples were obtained from each bin with size equal to that of the smaller bin (in blue). A binomial distribution with p=0.5 is shown in grey for reference.

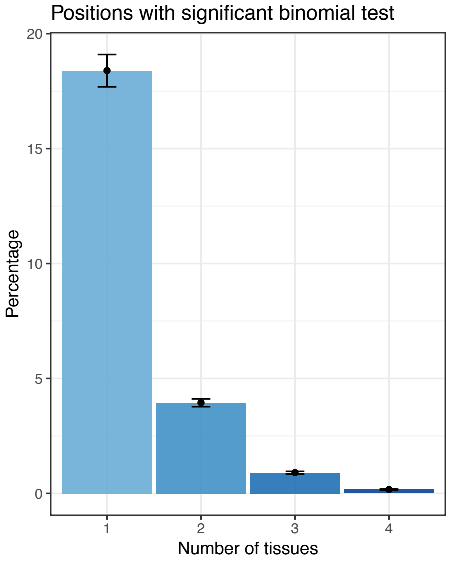


Figure 33. Power to detect heterozygous variants by number of tissues. Median percentages of high-confidence heterozygous positions that would be classified as not heterozygous because their binomial test in all the 1, 2, 3 or 4 tissues tested is significant (p-value<0.05). Black bars show they standard deviation. For each number of tissues, all possible comparisons were performed.

Number of haplotypes

True somatic variants create a third haplotype in the region, i.e., the maternal or the paternal haplotype suffer a mutation, creating a new haplotype with their previous haplotype along with that mutation. For this reason, somatic variants should exclusively be in phase with one of the paternal haplotypes. However, artefacts affect both the maternal and paternal haplotypes, creating at least four haplotypes with a nearby heterozygous variant (Fig. 34). Because we use short reads, only a few variants can be phased, but we can use that information to detect false positives. We annotated it with our custom python script, which allowed us to remove variants with four haplotypes or more.

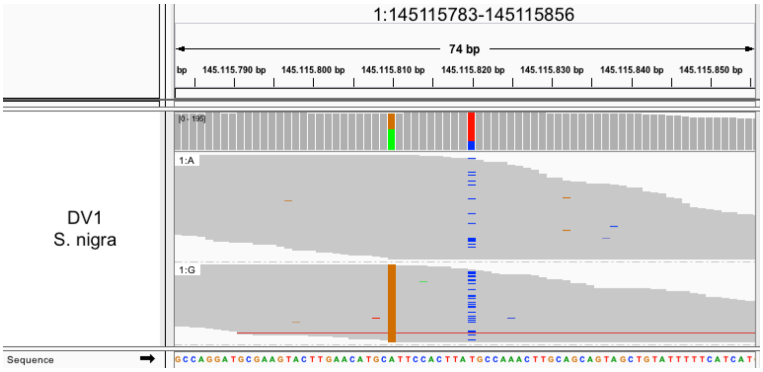


Figure 34. Example of a region with four haplotypes. Considering the A>G (green and brown) variant as a heterozygous one, the candidate somatic variant, a C>T substitution (red and blue), should be in a proportion of the reads carrying either A or G at the heterozygous position. However, there are reads whose haplotype is AT, AC, GT and GC.

Exome sequencing considerations

Exome sequencing by capture consists of the retention of DNA fragments that hybridize to the exome probes. Variants in a region overlapping a probe will reduce the efficiency of the hybridization, reducing the number of fragments carrying the variant that are retained. This implies that at heterozygous positions, the alternative allele will have a smaller frequency than the reference allele, which is known as reference bias. As with the determination of heterozygous positions, having five tissues gives us more power to find the true frequency of a variant. Also, reference bias is more relevant when there are multiple variants close to each other. Because these would be clustered mutations, which we do not consider in the first place, this should not be an issue for us.

More, exome capture results in target regions covered at the expected depth and adjacent regions being covered by reads generated from the fragments that partially overlapped the probes, and coverage decreasing with distance from target regions. Intersecting BAMs with the target-region bed creates the same effect *in silico*: reads partially overlapping the specified regions are retrieved, getting positions outside the target region covered. When inputting this data into a caller, variants are also called outside the target region. Because the coverage is much lower in these regions, calls were only considered if they were on the target region. To remove them, they were first annotated in the VCFs.

3.2. Candidate somatic variants with VarScan 2

Some of the abovementioned features completely prevent us from calling somatic variants in a set of genomic positions: those overlapping our somatic "non-callable" track, overlapping a CNV, close to indels, by homopolymers and off-target positions. However, many other features do not imply a binary decision, but give us important information. The probability of a candidate variant passing a test is higher if it is truly a somatic variant, but the tests cannot perfectly separate false positives from true positives (see Fig. 31 for an example). Since we are testing multiple features — number of reads supporting the alternative allele, multiple alleles, the number of samples with alternative reads, R1/R2 bias, clustered variants, PIR, strand bias, heterozygosity and number of haplotypes when possible — it is extremely unlikely that all tests will be passed for all tissues, even for a true somatic variant.

Also, requiring at least 2 reads supporting the alternative allele in a sample with a coverage of 60x puts our sensitivity limit at a variant allele frequency of 3%, or at 2% if coverage is at our upper threshold, 100x. Assuming equal contribution of cells to the gastrula, the cell division at which those mutations occurred can be calculated with formula 1.

$$Cell\ division = log2\left(\frac{1}{(AD/DP)}\right)$$

(Formula 1)

Where AD is the number of reads supporting the alternative allele and DP is the total depth at a position. Their ratio is the variant allele frequency, and its inverse is the number of existing cells at the moment the mutation happened. If we consider a mutation happens in replication, so that only one of the daughter cells inherits the mutation, its log2 gives us the division number at which the error occurred. For our DP range (20-100), with an AD of 2, it would be between the 3rd and 6th division. Because those are very early cell divisions, happening even before the appearance of the blastocoel, those variants will be present in every adult organ, probably at different proportions because of stochasticity and clonal expansion in certain tissues. Besides epithelia, one of the tissues where clonal expansion is highly recognized at is blood.

It follows that two different strategies are needed to get candidate variants. To pick up variants common to all tissues, we required a position to pass each of the filters in at least 4 tissues, not demanding that they are the same 4 tissues for each filter. To identify variants exclusive to a tissue, be it due to clonal expansion in blood or to sampling or other type of stochasticity, a variant had to pass each filter in a single tissue.

Somatic variants common to multiple tissues

To filter variants, we used the information VarScan 2 provided as well as that we added to the VCFs. Specifically, we required at least 4 tissues to pass these filters:

1. Not multiallelic
2. Not overlapping somatic non-callable tracks
3. On target
4. Not overlapping a CNV call and DP 20x to 100x
5. No more than 3 variants close by in any of the individuals' tissues
6. Not within 5 bp of indels
7. Not by homopolymers
8. AD >=2
9. At most called in 1 sample from another individual
10. R1 and R2 proportion between 0.25 and 0.75 for each allele
11. Non-significant FET for strand and allele
12. Strand ratio between 0.5 and 2
13. All tissues with significant binomial VAF test
14. At most 3 haplotypes
15. Unbiased position in reads for both alleles

Applying these filters, we got 3 candidates (table 3). All of them were present in the four central nervous samples, and two of them were also present in blood, even if at lower frequencies. Each tissue has a quite low AD, showing the power of combining the information from multiple tissues.

Although all of them have a dbSNP ID, they are present in 1 to 3 samples in gnomAD and/or TOPmed, which makes their population frequency extremely low. Two of them are missense variants in the genes *PCDH10* and *DENND4A* and the third one overlaps the UTR regions of two genes. All of the changes have high CADD scores, indicating their functional relevance.

PCDH10 is expressed in brain and arteries (The GTEx Consortium 2013) and is essential for normal forebrain axon outgrowth (Williamson et al. 2007). It was found in homozygous deletion in a study of consanguineous autism spectrum disorder (ASD) families (Morrow et al. 2008) where they demonstrated that *PCDH10* was strongly up-regulated in hippocampal neurons in response to membrane depolarization and that it is a transcriptional target of *MEF2*, a transcription factor induced by neuronal activity.

The other missense variant affects *DENND4A* a gene involved in vesicle-mediated transport (Belinky et al. 2015) which was related to Parkinson's in a translatome-regulatory network analysis. *DENND4A* was found to be one of 19 genes driving the expression signature change of dopaminergic (DA) neurons after Parkinson induction with *MPTP* (Brichta et al. 2015). This specific variant is predicted as deleterious and probably damaging by SIFT and PolyPhen respectively.

The last variant overlaps with two genes in the two strands. On the forward strand, the variant is in the 5' UTR of *PACRGL*, the Parkin Coregulated Like gene, and it is predicted by Ensembl (Hunt et al. 2018) to activate non-sense mediated decay, a route involved in the reduction of expression of aberrant proteins. This gene was found to be regulated by the same promoter as that of *PARK2* (West et al. 2003). On the reverse strand it is the first base pair of the 3' UTR of the *KCNIP4*, a gene that encodes a K channel-interacting protein, which modulates the activity of Kv4 A-type potassium channels thus playing a significant role in the firing of action potentials within the neurons (Holmqvist et al. 2002). A study on the regulatory networks in Parkinson disease (Su et al. 2018) identified a lncRNA whose target is *KCNIP4*, making both genes interesting candidates.

Table 3. Somatic variants common to multiple tissues. Chromosome and position are in reference to hg19. VAF: Variant allele frequency. AD: Alternative allele depth.

	Individual	DV1	DV6	DV6
	Chr	4	4	15
	Position	134073018	20731704	66044838
	dbSNP	rs754282504	rs1224107540	rs1268304474
	Frequency	<1e-4	<1e-4	<1e-4
	Consequence	Missense	3 prime UTR/ Intron NMD	Missense
	Gene	*PCDH10*	*KCNIP4/ PACRGL*	*DENND4A*
	CADD	22.6	18.08	33
	Reference	G	G	G
	Alternative	C	A	A
VAF	Blood	1.61%	0%	5.26%
	Cerebellum	13.64%	4.17%	10.61%
	Striatum	6.9%	5.88%	9.84%
	Neocortex	9.57%	5.26%	16.98%
	Substantia nigra	13.93%	6.67%	12.5%
AD	Blood	1	0	4
	Cerebellum	9	2	7
	Striatum	4	3	6
	Neocortex	9	3	9
	Substantia nigra	17	3	8

Tissue-exclusive somatic variants

Looking exclusively at read features from one tissue constitutes a rougher approach. We have to be stricter because we do not have the information added by the presence of the same variant in the other tissues with features indicating good quality. For this reason, we increment the minimum AD from 2 to 5, the AD at which we see a reduction of random noise (Fig. 21). As for an upper AD threshold, we know no specific threshold can be successful in removing heterozygous variants (Fig. 31). However, we can use the information provided by the other tissues to remove those cases; if a tissue shows a VAF compatible with heterozygosity, most probably the variant will be heterozygous. Also, requiring it not to be in the dbSNP common variant database is an obvious step to remove heterozygous variants.

To filter each tissue independently we required multiple conditions. Most of them are the same as for shared variants, and the differences are highlighted in bold:

1. Not multiallelic
2. Not overlapping somatic non-callable tracks
3. On target
4. Not overlapping a CNV call and DP 20x to 100x
5. No more than 3 variants close by in any of the individuals' tissues
6. Not within 5 bp of indels
7. Not by homopolymers
8. **AD >=5**
9. At most called in 1 sample from another individual
10. R1 and R2 proportion between 0.25 and 0.75 for each allele
11. Non-significant FET for strand and allele
12. Strand ratio between 0.5 and 2
13. All tissues with significant binomial VAF test
14. At most 3 haplotypes
15. Unbiased position in reads for both alleles
16. **Not in dbSNP common variants**

From this we obtained 10 variants (table 4). Two of those variants (in grey) are present in the 5 tissues and as expected are also in table 3. The one on *PCDH10* was recovered from two different tissues while the one on *DENND4A* only passed filters for the neocortex sample. From the remaining 8 variants, 5 are blood exclusive, 3 are mainly present in blood with 1 read supporting the alternative allele in substantia nigra or striatum, and the other one is neocortex exclusive.

Inspecting these variants in IGV (J. T. Robinson et al. 2011) some reads whose mate maps too far away can be identified. The reads carrying the alternative allele look fine, but orthogonal validation is necessary to confirm they are true variants. However, blood suffers from clonal expansion (Champion et al. 1997; Zink et al. 2017), therefore it is reasonable that variants are exclusive to this tissue or have higher or lower frequencies than the other tissues depending on whether the expanded lineages carry that variant or not. This is what we observe, giving credibility to our results.

Table 4. Somatic variants identified in a single tissue. Chromosome and position are in reference to hg19. VAF: Variant allele frequency. AD: Alternative allele depth. Variants with information in grey were also recovered with the previous strategy.

	Sample	DV1B	DV1C, DV1N	DV3B	DV3B	DV3B	DV4N	DV6B	DV6N	DV7B	DV8B
	Chr	12	4	5	12	13	2	2	15	12	11
	Position	93873233	134073018	94990053	66849194	21987914	158115786	145157076	66044838	57976385	58170304
	dbSNP	rs1201602329	rs754282504	rs767559079	rs1296372237	NA	NA	rs730881194	rs1268304474	NA	rs763347958
	Frequency	<1e-4	<1e-4	0	0	0	0	<1e-3	<1e-4	0	<1e-4
	Consequence	Synonym./NMD	Missense	Synonymous	Missense	Intronic acceptor site "synonymous"	Missense	Missense	Missense	Missense	Synonymous
	Gene	*MRPL42*	*PCDH10*	*RFESD*	*GRIP1*	*ZDHHC20*	*GALNT5*	*ZEB2*	*DENND4A*	KIF5A	OR5B3
	CADD	12.09	22.6	16.80	29.1	12.41	14.28	23.8	33	34	0.541
	Reference	A	G	A	C	A	G	G	G	G	G
	Alternative	G	C	G	A	G	A	A	A	A	A
VAF	Blood	20%	1.61%	14.93%	13.56%	18.75%	0%	12.77%	5.26%	8.96%	5.75%
	Cerebellum	0%	13.64%	0%	0%	0%	0%	0%	10.61%	0%	0
	Striatum	0%	6.9%	0%	0%	0%	0%	0%	9.84%	1.75%	0
	Neocortex	0%	9.57%	0%	0%	0%	6.25%	0%	16.98%	0%	0
	Subst. nigra	0%	13.93%	0%	1.64%	0%	0%	0%	12.5%	0%	0
AD	Blood	6	1	10	8	6	0	6	4	6	5
	Cerebellum	0	9	0	0	0	0	0	7	0	0
	Striatum	0	4	0	0	0	0	0	6	1	0
	Neocortex	0	9	0	0	0	5	0	9	0	0
	Subst. nigra	0	17	0	1	0	0	0	8	0	0

The first blood exclusive somatic variant overlaps the gene *MRPL42*. It is a previously observed variant with very low population frequency. *MRPL42* is a mitochondrial ribosomal protein, involved in mitochondrial translation. Ensembl predicts it to induce non-sense mediated decay in some transcripts, but it is a synonymous variant for the main transcripts.

A second blood exclusive synonymous variant, was found on the *RFESD* gene, involved in oxidation-reduction processes. Although variants at that position have been reported, none of them were A>G. Another synonymous variant was found at gene *OR5B3*, an olfactory receptor gene.

An intriguing variant is that on *ZDHHC20*. In this case it is an A>G change but because the gene is in the reverse strand, its relevance would be caused by the T>C change in that strand. It is the third base upstream of an exon, so part of the splicing acceptor site, which changes from TAG to CAG, which still fits the consensus sequence of pyrimidine-A-C. The position is not reported as variant in any population sequencing project. A study on the effect of glial cell line-derived neurotrophic factor (GDNF), a transforming growth factor involved in the development and maintenance of mesencephalic DA neurons (Lin et al. 1993), over microRNAs in MN9D mouse cells, found it to result in the differential expression of 143 miRNAs, 3 of them targeting *ZDHHC20* (L. Li et al. 2013).

The remaining 4 variants are missense variants. The one on *GRIP1*, a glutamate receptor interacting protein, is found in blood and also has a supporting read in the substantia nigra. This gene supplies synapses with two key synaptic proteins, GluA2 and N-cadherin, by acting as a scaffold at trafficking vesicles (Heisler et al. 2014). The one on *GALNT5*, exclusively found in the neocortex sample, is a missense variant not reported in population studies either. The predicted CADD is moderately high and the protein is involved in glycan biosynthesis and protein metabolism, and is causative of hereditary multiple exostoses (Simmons et al. 1999).

The missense variant on gene *ZEB2* was only found in blood. It has been reported previously at very low frequency and has a moderately high CADD score, SIFT prediction of deleterious and PolyPhen as possibly damaging. This zinc finger homeodomain protein is responsible for Mowat-Wilson syndrome. Also, it is a negative regulator of midbrain dopaminergic axon growth and target innervation (Hegarty et al. 2017), acts in myelination (Weng et al. 2012) and regulates the fate switch between cortical and striatal interneurons (McKinsey et al. 2013).

Finally, a missense variant in *KIF5A* was found in blood and striatum samples. It is a member of the kinesin family mainly expressed in neurons (Niclas et al. 1994), where it acts as a microtubule-dependent motor required for slow axonal transport of neurofilament proteins (Hirokawa et al. 2009) whose mutation causes monogenic spastic paraplegia (Reid et al. 2002), Charcot-Marie-Tooth disease type 2 (Y.-T. Liu et al. 2014) and familial amyotrophic lateral sclerosis (Brenner et al. 2018). A missense mutation in this same gene has been previously described in a Parkinson patient (Martikainen et al. 2015) and its expression was increased during progression of dementia associated with PD (Stamper et al. 2008). This specific missense variant has a high CADD, indicating possibly damaging consequences.

Tissue-exclusive somatic variants without other tissues information

If we use the same exact filters as for the previous analysis but exchange the binomial test result in all tissues by a harsh threshold at VAF of 20%, which we derive from Fig. 31, we get the same 8 variants we got before (table 4) plus two more (table 5).

The first one, on the *EDAR* gene, looks actually like a heterozygous variant when we look at the other tissues. Nonetheless, the variant is predicted by most methods as damaging and this gene is responsible for ectodermal dysplasia as well as hair thickness in Asian populations.

The other one overlaps with one of the transcripts of the gene *SYT15*, where it supposes a missense variant. Maybe more importantly, it overlaps with its promoter. This protein, involved in the synaptic vesicular cargo trafficking was affected by a CNV in a study on Parkinson disease patients (La Cognata et al. 2017). It is interesting that all tissues have frequencies between 16 to 36%. Because of this, not all of them pass the binomial filter, but because also Haplotype Caller with ploidy 10 (see below) reports similar frequencies, it seems plausible that it is a somatic variant.

Table 5. Somatic variants identified in a single tissue without other tissue information. Chromosome and position are in reference to hg19. VAF: Variant allele frequency. AD: Alternative allele depth.

	Sample	DV5B	DV9S
	Chr	2	10
	Position	109524434	46970793
	dbSNP	rs757685532	rs752701929
	Frequency	<1e-4	8,00E-03
	Consequence	Missense	Missense/ TF binding site
	Gene	*EDAR*	*SYT15/ TBX15, TBX20, TBX1*
	CADD	26.8	3.674
	Reference	C	G
	Alternative	T	A
VAF	Blood	19.23%	36.36%
	Cerebellum	34.29%	25.4%
	Striatum	40.91%	16.67%
	Neocortex	41.18%	26.19%
	Substantia nigra	56%	18.6%
AD	Blood	5	12
	Cerebellum	12	16
	Striatum	9	5
	Neocortex	14	11
	Substantia nigra	14	8

3.3 Candidate somatic variants with HaplotypeCaller ploidy 10

Samples were also called with HaplotypeCaller. Although it is a germline caller, it is designed to be applicable to higher ploidy organisms by changing its allele frequency expectations. To increase sensitivity, we set the ploidy parameter to 10 (default is 2). This way, the priors change, increasing its sensitivity. We annotated the VCFs with the same information we did on VarScan 2 VCFs.

Somatic variants common to multiple tissues

Using exactly the same filters we did with VarScan 2 VCFs to get variants common to multiple tissues, we got the exact same 3 variants (table 6). Frequencies also seem very comparable between the two.

Table 6. Somatic variants common to multiple tissues from Haploype Caller. Chromosome and position are in reference to hg19. VAF: Variant allele frequency. AD: Alternative allele depth. HCP10: Haplotype Caller with ploidy parameter set to 10.

	Individual	DV1		DV6		DV6	
	Chr	4		4		15	
	Position	134073018		20731704		66044838	
	dbSNP	rs754282504		rs1224107540		rs1268304474	
	Method	VarScan 2	HCP10	VarScan 2	HCP10	VarScan 2	HCP10
VAF	Blood	1.61%	0%	0%	0%	5.26%	4.40%
	Cerebellum	13.64%	13.89%	4.17%	5.46%	10.61%	10.26%
	Striatum	6.9%	8.96%	5.88%	5%	9.84%	9.86%
	Neocortex	9.57%	10.20%	5.26%	4.48%	16.98%	20%
	Subst. nigra	13.93%	14.93%	6.67%	5.26%	12.5%	14.81%
AD	Blood	1	0	0	0	4	4
	Cerebellum	9	10	2	3	7	8
	Striatum	4	6	3	3	6	7
	Neocortex	9	10	3	3	9	12
	Subst. nigra	17	20	3	3	8	12

Tissue-exclusive somatic variants

Because is not possible to make HaplotypeCaller as lax as VarScan 2, when a candidate SNV is present in an individual's tissue with AD>=5 and the same variant is present in a handful of other samples with 1 or 2 reads per sample, which would indicate some kind of bias, HaplotypeCaller would not call those other lower AD samples, making it difficult for us to detect the false positive. To try to circumvent that, we called all 50 samples together. However, after using the same filters we used with VarScan 2, we found many passing variants were supported by reads in multiple tissues of different individuals but were only called in the most confident sample by HaplotypeCaller. This makes sense for the caller, it is applying a confidence score to remove those cases that are very clearly false positives, but unfortunately leaves us powerless to remove the higher confidence false positives.

We explored whether any HaplotypeCaller quality fields could remove those cases, but we did not find any. Of note, certain quality scores have to be treated differently depending on ploidy and coverage. Genotype quality (GQ) in HaplotypeCaller is calculated as the difference between the two lowest phred-scale likelihoods for each genotype. When ploidy is 2 there are three possible genotypes, so for coverages well above 20x, the difference between their probabilities should always be high. A threshold at GQ>=90 is therefore sometimes used in this scenario (Horai et al. 2018). However, when setting ploidies as high as 10, the probabilities of similar genotypes (e.g. 0/0/0/0/0/0/1/1/1/1 vs 0/0/0/0/0/1/1/1/1/1) are quite similar, producing GQ values invariantly low.

If we use VarScan 2 to filter those cases out, we get 16 variants passing filters. Once again, we are able to retrieve 2 out of the 3 variants common to multiple tissues (grey in table 4) as well as all the other 8 variants we got with VarScan 2 filtering per tissue (black in table 4). We also get 6 more variants. After IGV inspection, one of them looked too strand biased, so it was discarded. The remaining 5 variants are shown in table 7.

One of the variants was not detected in VarScan 2 because it was triallelic, which belongs to the set of noisier calls. Two others had less than 5 AD after VarScan 2 internal read filtering and the remaining two had more than 4 variants called within read distance, but IGV inspection shows the other variants are actually noise we are taking into account with VarScan 2. This shows that HaplotypeCaller improves sensitivity and its specificity can be improved by using VarScan 2 calls and very probably also just by looking for alternative reads in other samples directly on the BAM files with tools such as pysam.

As for the newly discovered variants, they were all detected in blood but two of them have also support in the striatum and one has it in the substantia nigra too. The first variant is a missense mutation in the gene *ZSCAN16*. It is a zinc protein whose function is not well characterized. A deep intronic variant was also detected in *NAV3*, the neuron navigator 3 gene, named after its role in axon guidance was determined in *Caenorhabditis elegans* (Maes, Barceló, and Buesa 2002). In a study of circulating cell-free microRNAs in Parkinson's patients, miR-29a was found to be downregulated in PD cases (Botta-Orfila et al. 2014) and has also been seen aberrantly expressed in Alzheimer's (Batistela et al. 2017). *NAV3* is a target of this miRNA (Batistela et al. 2017), so it would be upregulated in PD. Also, a copy number variant affecting the gene has been found in PD cases (Botta-Orfila et al. 2014).

Table 7. Somatic variants identified in a single tissue by HaplotypeCaller exclusively. Chromosome and position are in reference to hg19. VAF: Variant allele frequency. AD: Alternative allele depth.

	Sample	DV3B	DV3B	DV7B	DV7B	DV10B
	Chr	6	12	6	9	5
	Position	28097592	78521098	31905132	87367003	55264231
	dbSNP	rs766950970	rs968223137	rs765550448	NA	NA
	Frequency	<1e-4	<1e-4	<1e-4	0	0
	Consequence	Missense	Intronic	Missense	Splicing donor site	Intronic
	Gene	*ZSCAN16*	*NAV3*	*C2*	*NTRK2*	*IL6ST*
	CADD	19.73	2.262	22	21.6	9.827
	Reference	G	C	C	A	G
	Alternative	A	T	T	G	T
VAF	Blood	9.37%	8.93%	29.55%	28.28%	8.05%
	Cerebellum	0%	0%	0%	0%	0%
	Striatum	0%	0%	3.51%	4.32%	0%
	Neocortex	0%	0%	0%	0%	0%
	Subst.nigra	0%	0%	0%	3.79%	0%
AD	Blood	6	5	26	28	7
	Cerebellum	0	0	0	0	0
	Striatum	0	0	2	6	0
	Neocortex	0	0	0	0	0
	Subst. nigra	0	0	0	5	0

A missense variant quite frequent in blood affects *C2*, a gene involved in the immune system. Studies have looked at the association of variants in this and other HLA-linked complement markers, finding no association (Nerl, Mayeux, and O'Neill 1984).

An unreported variant in *NTRK2* is at the splicing donor site of the 14th exon. It is the 3rd base so it does not affect the consensus sequence GU. Nonetheless, this gene is a receptor of neurotrophic factors, which have been proposed to have a role in neurodegeneration (Dawbarn and Allen 2003). Specifically, in a primate MPTP model of PD, infusion with a neurotrophic factor reversed motor dysfunction (Grondin et al. 2002). It was also found to be down-regulated in Alzheimer disease brains (Ferrer et al. 1999). Finally, an intronic variant was found at gene *IL6ST*, an interleukin signal transducer found to be differentially expressed in a mouse MPTP model (L. Gao et al. 2015).

Summarizing all the results, at least one somatic variant was found at each individual (tables 3-7) and the involved genes had been previously related to the nervous system or even PD for 6 of the patients. Also, all but DV7 were heterozygous for a variant in genes previously related to PD (table 2).

3.4 Tissue clustering by somatic variant allele frequencies

The allele frequency of somatic mutations generated during embryonic development will differ among adult tissues depending on the proportion of mutant cells present in the founder cell population of each tissue. Development and growth dynamics of each organ as well as clonal expansion during adult tissue maintenance will also alter the final frequencies. Hence, distance among the tissues can inform us about these processes.

The frequencies at each tissue from every somatic mutation detected were accumulated. Tissues were clustered by Pearson correlation based on the variant allele frequencies (Fig. 35). Blood was the most distant tissue. This was expected since out of the 18 somatic variants, 12 are exclusive to blood. The distance among the four central nervous system tissues is thus very small. Still, the distance between the two basal ganglia structures is smaller, but this is mainly driven by the two last mutations, those on *EDAR* and *SYT15*, whose status as somatic variants is less clear. Higher sensitivity or more individuals could help us to define these relationships with better accuracy.

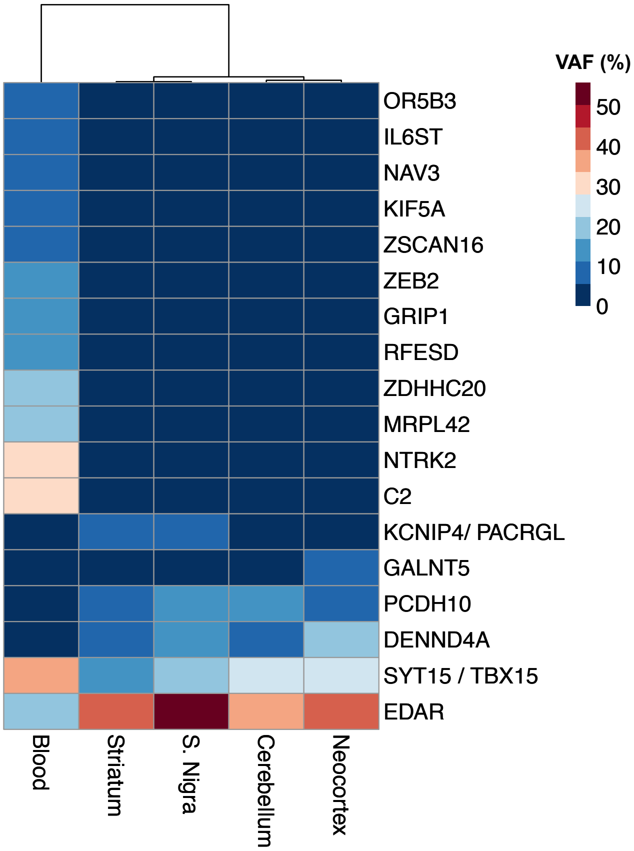


Figure 35. Tissue clustering by somatic mutation frequencies. Heatmap values indicate the variant allele frequency of the variants from tables 3-7 at each tissue. The top dendrogram resulted of Pearson correlation clustering based on the frequencies.

Exploring somatic copy number variants in Alzheimer disease with array CGH data

1. Array CGH data processing

Array comparative genomic hybridization (aCGH) technology was used to explore somatic copy number variation in Alzheimer disease (AD). Matched blood and hippocampus DNA samples were obtained from 21 AD patients, 2 vascular dementia (VaD) patients and 3 controls (table S3). Samples were processed in two different batches. Each tissue's DNA was hybridized to the Roche Exon-Focused aCGH together with a commercially available control genome, the male human genomic DNA from Promega.

Quality control

The control genome was labelled with the red fluorescent dye cyanine 5 whereas each test genome was labelled with a green fluorescent dye, cyanine 3. However, a PCA of the raw data (Fig. 36) showed more variance among the red than among the green channel at both batches.

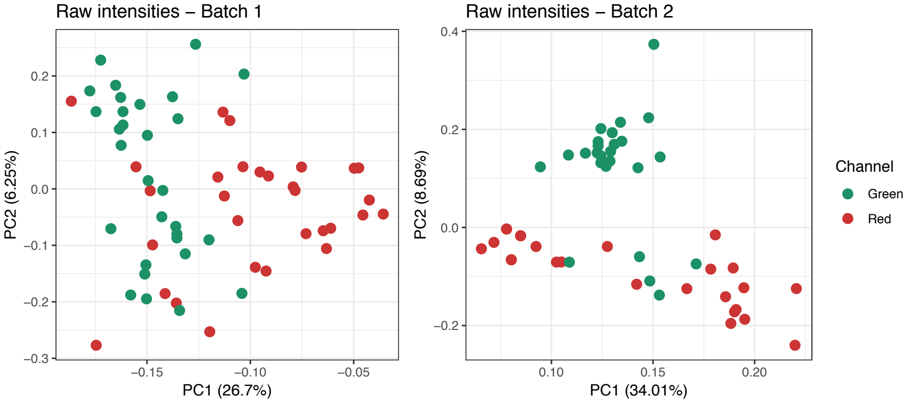


Figure 36. PCA of each channel raw intensities. The two first principal components for batch 1 (left) and batch 2 (right) are shown, with the amount of variance explained by each of them in parenthesis.

Because the variability in the raw light emissions strongly depends on the consistency of the excitation light, regions with apparent germline copy number variants were used to confirm the labelling. Log2 ratios between the two channels by genomic position were inspected for both the blood and hippocampus of two samples (Fig. S9). This allowed the detection of regions with extremely low log2 ratios in just one individual and present in both its tissues. Median intensities for each channel showed that the most variable was the green channel (table 8), confirming this was the test sample channel.

Table 8. Raw intensities at deletions. Raw green and red channel intensities for regions where log2 ratios were extremely low. Grey numbers highlight the smallest values at each genomic region, showing that the green channel is variable among individuals and less between tissues.

			Raw intensity		
			1:118940915-119024481	**1:204588543-204828734**	**20:5938122-6021265**
1G	Hippocampus	Red	2062.47	2206.17	1754.12
		Green	3241.37	3762.1	412.43
	Blood	Red	1375.45	1492.27	1128.31
		Green	3078.73	3715.92	363.65
2M	Hippocampus	Red	1519.49	1675.49	1012.02
		Green	352.82	354.25	2098.71
	Blood	Red	3828.96	3701.14	2525.84
		Green	313.76	369.13	2767.94

Moreover, densities of log2 ratios at the sex chromosomes (Fig. S10) showed that the distribution of chromosome X was either centered around 0, corresponding to the same copy number as the control, or around a mean of 0.65, indicating a duplication with respect to the control. Correspondingly, chromosome Y was either centered around 0 or dispersed towards negative log2 ratios, with a mean of -1.64, indicating a deletion with respect to the control. Since the control sample is a male, this further confirmed the channel labelling. Also, both tissues of all samples showed consistent genomic sex.

Normalization

As expected, extensive differences are found between the median and dispersion of raw light intensities of the different channels and arrays (Fig. 37). MA plots were used to examine the common bias in the log2 ratios (M) with respect to the mean intensity (A). Indeed, higher light intensities showed higher log2 ratios (example in Fig. 38), a tendency that affected different arrays with variable strength (Fig. S11).

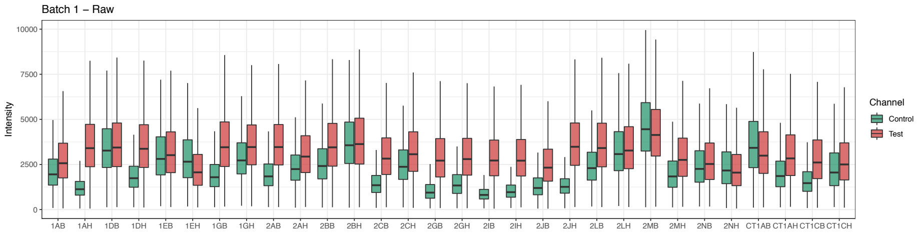


Figure 37. Raw light intensity distributions. Boxplot showing the raw light intensity per each channel and sample from batch 1. Individual names are followed by a "B" for blood samples and "H" for hippocampus samples.

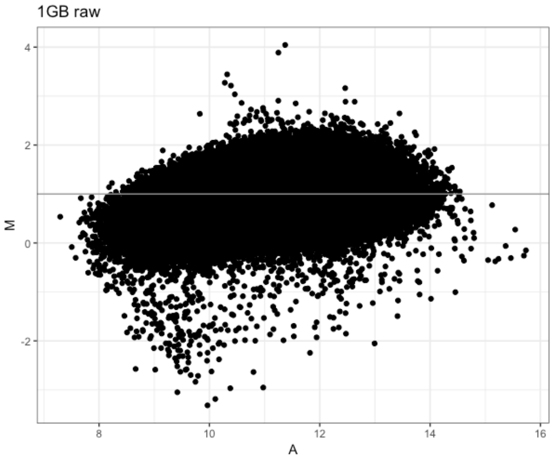


Figure 38. MA plot of raw data. Mean intensity of both channels (A) against log2 ratio (M) at each probe in the blood sample from individual 1G. Grey line is at the mode of the M distribution.

Accordingly, we applied quantile normalization to the raw data. Since the variance of the two channels is quite different, we normalized the intensities from both channels from all arrays together. Log2 ratios after normalization were centered at 0 with outliers were still identifiable, even those with less extreme log2 ratios (Fig. 39), which could be the regions were only a portion of the cells have a copy number variant and those we are most interested in.

On the other hand, batch effect correction produced strong deviations for a subset of probes at some arrays (Fig. S12). Since the number of arrays in each batch is sufficient for independent analysis, we restrained from performing batch correction and kept the two datasets separated in the subsequent processes.

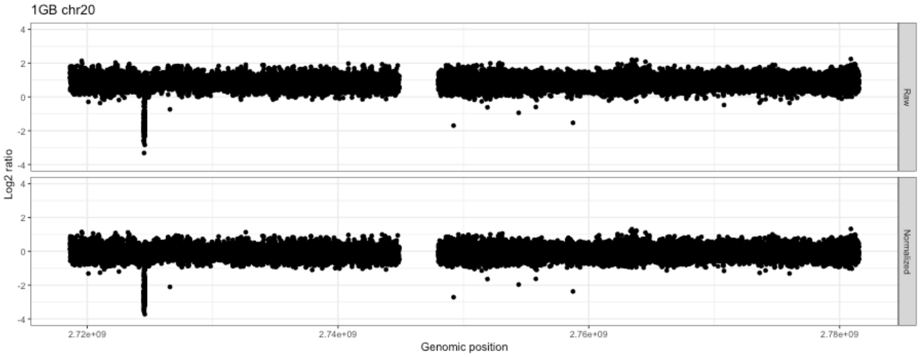


Figure 39. Log2 ratios before and after quantile normalization. Log2 ratios per position in the chromosome 20 are shown for 1G's blood before and after quantile normalization.

Alternative normalization approaches exist and are mainly used in cancer studies (Staaf et al. 2007). Their objective is to normalize the data while considering the existence of aneuploidies, trisomies or considerably sized rearrangements. Because these extreme regions can affect quantile normalization, they are excluded for the centralization of the data. However, initial inspection of our data, with log2 ratios vs position, did not show any of such rearrangements, and since quantile normalization was successful at keeping shorter variants (Fig. 39), we decided to use the latter approach.

2. Array CGH data copy number calling

Segmentation and calling

Normalized log2 ratios were segmented with the package DNAcopy (van de Wiel et al. 2007). We tested different SD thresholds for merging adjacent regions: 1, 2 and 3. That is, the distribution of log2 ratios is inspected in windows by the software. Then, adjacent windows whose log2 ratio mean is within the selected SD limit of the original window are merged to it. Inspection of the results obtained with the different values showed that segmentation with SD=1 and SD=2 resulted in too many short fragments, which would be difficult to compare between the tissues in subsequent analysis. On the contrary, segmentation with SD=3 reflected the raw log2 ratios variance while limiting the amount of noise incorporated (Fig. 40), so this was the selected parameter.

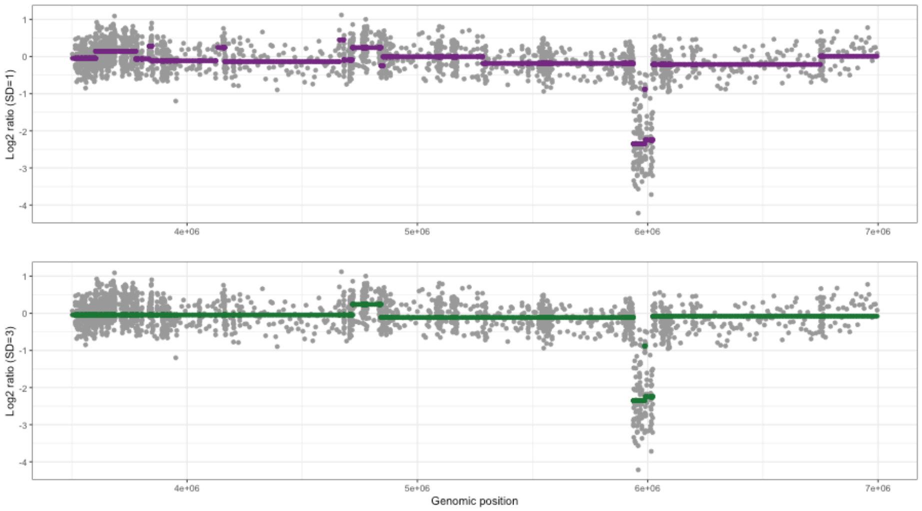


Figure 40. Segmented log2 ratios with different SD parameters. Normalized log2 ratios at each probe, in grey, were segmented with SD=1, resulting in the purple values. When segmented with SD=3, the green values were obtained.

Copy number variant probabilities were called with CGHcall (van de Wiel et al. 2007), which calls aberrations for aCGH data using a six-state mixture model. In short, the fragmented signal from the previous step is used to find breakpoints. Most algorithms assume three possible copy number states: duplication, normal or deletion. However, CGHcall incorporates two more classes, differentiating between one and two copies lost or gained, which improves the detection of single copy gains and amplifications. The output consists on the probabilities each probe has to belong to each of the six states as well as calls indicating the most probable copy number.

Germline copy number variants

Segmentation and calling quality were evaluated by comparing the correlations of autosomal probes' log2 ratios between arrays belonging to the same individual and to different individuals after each step (Fig. 41). The median of the correlations between normalized log2 ratios of tissues from the same individual was higher than when comparing tissues from different individuals but their ranges overlapped considerably. After segmentation with DNAcopy, the ranges separated better but still overlapped, probably indicating noise was still driving most of the correlations. Hence, probes were filtered by incorporating calling information. We required segments to have at least five consecutive probes with a CN call different than 0 and at least three probes with "extreme" normalized log2 ratios, that is, smaller than -1 for deletion calls and bigger than 0.585 for duplication calls, corresponding to CN 1 and 3, respectively. This produced much higher tissue correlations while keeping the individual comparison median at a similar level.

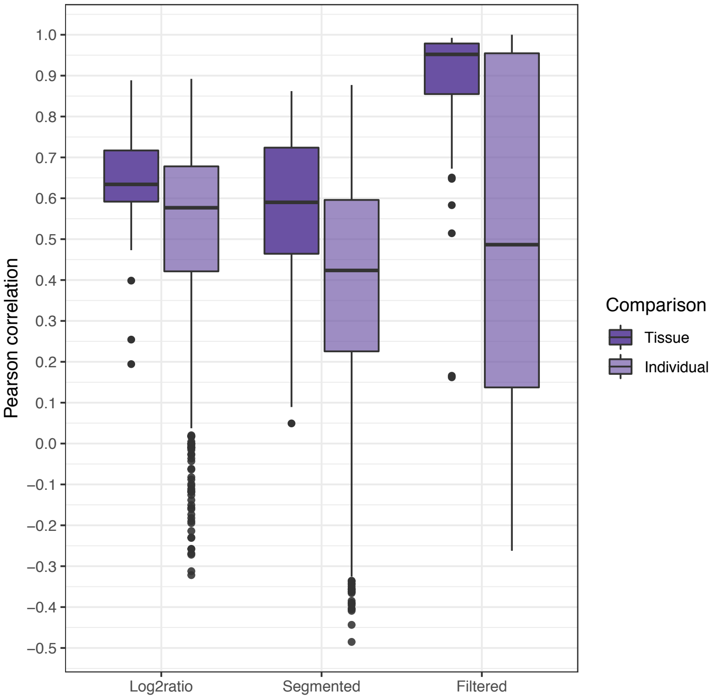


Figure 41. Distribution of Pearson correlations at each processing step. Autosomal probes correlation was calculated between the tissues of the same individual (dark purple) and for all possible comparisons within batch between different individuals (light purple).

This filter is quite restrictive, so we expected to only retrieve germline copy number variants together with those somatic variants that clonally expanded during tissue maintenance. Since blood experiences clonal expansion, if expanded somatic variants were called, we would expect a higher number of filtered segments in this tissue than in the hippocampus. Indeed, the median number of segments for blood samples was higher (Fig. 42), indicating some copy number variants present in a considerable number of cells are restricted to blood, at least at such high frequency.

Filtered segments were intersected with the RefSeq gene set. Those linked to Alzheimer disease in OMIM (table S4) were inspected with special attention. Of the six evaluated genes, only *APP* and *HFE* overlapped with filtered called segments. However, when comparing the log2 ratios and calls overlapping the filtered segments, many other samples presented similar patterns, as is the case for the samples of the first batch on the APP gene, where 12 out of 30 samples had a call made by CGHcall (Fig. 43). This could indicate that this gene is frequently altered in Alzheimer patients but nevertheless, out of the two control individuals, one of their tissues was also called in the same region. Furthermore, the number of samples called for a set of genes not linked to the disease but with the same number of filtered called probes was comparable (Fig. S13).

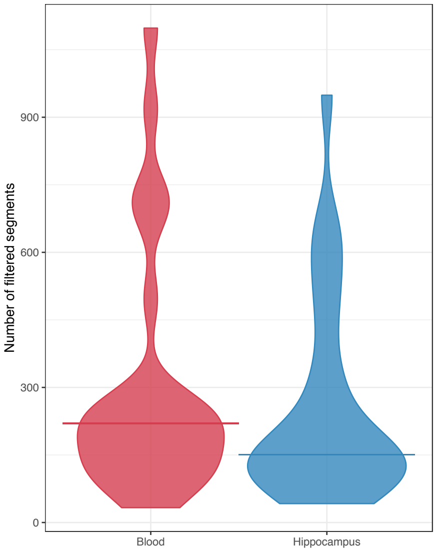


Figure 42. Number of filtered segments per tissue. The distribution of the number of segments filtered per sample by tissue. Horizontal lines show the median value for each set.

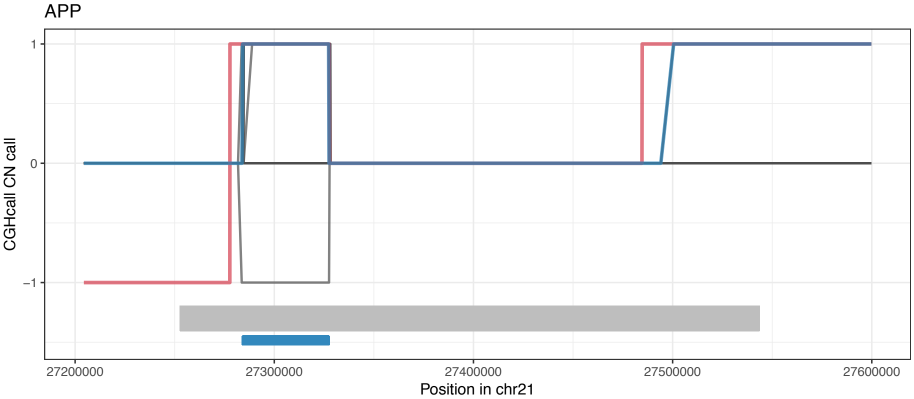


Figure 43. CGHcall calls at the *APP* gene. Each line shows the calls per sample at each position. The blue line corresponds to the sample with the filtered segment, 2IH, whereas the red line shows the calls for the blood of the same individual, 2IB. The grey rectangle highlights the gene position and the blue segment shows the region the filtered call overlapped.

Since no relevant calls clearly distinguishable from noise were found, we performed overrepresentation enrichment analyses (ORA) with the genes overlapping filtered calls from all the Alzheimer patients. When molecular function was evaluated with all protein coding genes as background, several processes were shown to be significantly enriched, mainly related to transcription regulator activity and nucleoside and ribonucleotide binding (Fig. 44). Proteins classified as nucleotide binding and purine ribonucleotide binding proteins were found to be downregulated in the hippocampus of Alzheimer patients (Ho Kim et al. 2015). Enrichment of OMIM's disease genes produced multiple significant results though Alzheimer disease was not one of them (Fig. S14).

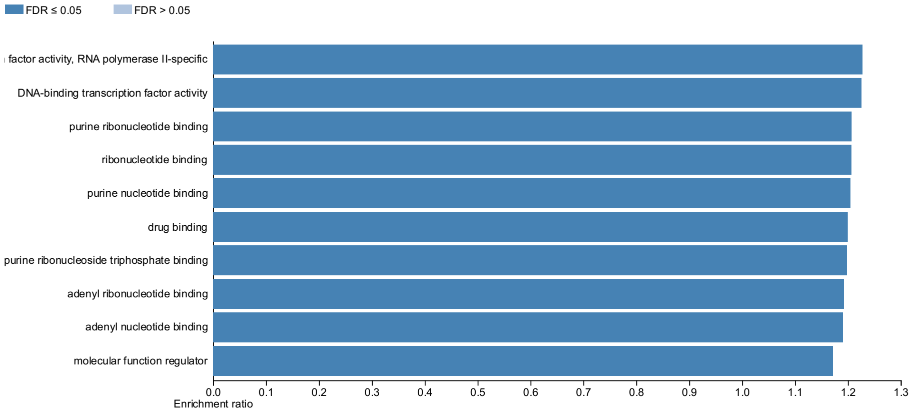


Figure 44. Filtered calls molecular function enrichment. Overrepresentation enrichment analysis for molecular function of genes overlapping filtered calls in the Alzheimer patients.

Somatic copy number variants

To explore somatic copy number variants in our data, we filtered the probes without considering CGHcall calls, as the thresholds this approach uses are designed for germline or clonally expanded variant identification. Hence, we selected segments with more than ten adjacent probes with segmented log2 ratios between -0.23 and -0.62, corresponding to 30% to 70% of cells with a deletion or between 0.2 and 0.43, roughly corresponding to the same proportion of cells carrying a duplication. As it was already difficult to differentiate between copy number 0 and 1 as well as between copy number 3 and 4, we focused on somatic copy alterations of a single copy, which are also more probable to occur.

We observed extremely low correlation between the tissues for this segments (Fig. 45), but tissue comparisons, that is, when we compare the segmented log2 ratios of both tissues from the same individual, still showed higher correlations than inter-individual comparisons, indicating more resemblance. Nonetheless, this small difference in log2 ratio to the control could be due to SNPs on the probes slightly affecting hybridization. Biologically, the lack of correlation could be explained by blood's clonal expansion. This extremely limited sensitivity for the somatic copy number range has been observed before (King et al. 2017).

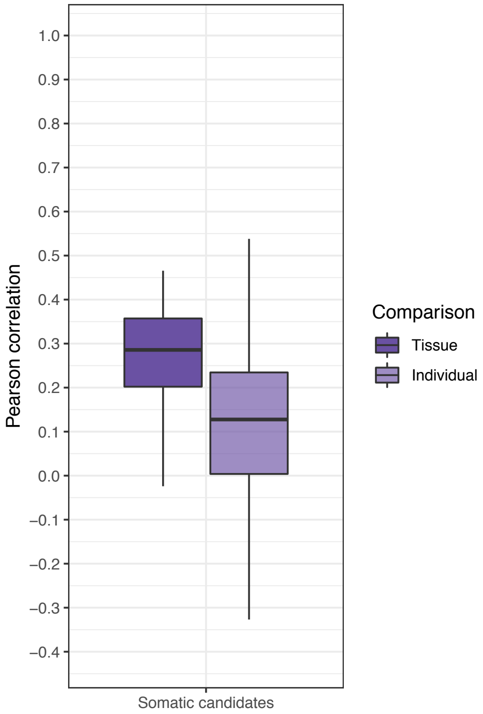


Figure 45. Distribution of Pearson correlations for candidate somatic copy number variants. Autosomal probes correlation was calculated between the tissues of the same individual (dark purple) and for all possible comparisons within batch between different individuals (light purple).

3. Comparison with whole genome sequencing

Because the increase in copy number we would expect from a somatic event is within the noise levels of the arrays, we used an orthogonal methodology to evaluate the replicability of the results. We sequenced the whole genome of blood and hippocampus from five of the individuals, two from the first batch and three from the second batch.

Comparison of copy number with mrCanavar

Copy number in 1kb windows was called with mrCanavar (see Methods), which uses coverage as a proxy of copy number. The caller identifies some windows as being of reference copy number, or the control regions. The copy number distribution of this control regions for each sample was comparable to the theoretical normal distribution calculated with the same mean and standard deviation, indicating the good quality in the results (Fig. S15). Although the copy number distribution in and out of control regions shows some overlap, they still are for the most part distinguishable (Fig. S16).

In order to compare WGS copy number to the arrays, log2 ratios were taken. Then, genomic windows corresponding to the previously identified putative somatic copy number mutations where selected. The medians of the log2 ratios of probes overlapping each of the WGS windows were calculated to allow a pairwise comparison. Of note, the Promega male genome used as a control in the arrays was not sequenced. This is because it is a variable sample derived from multiple anonymous donors, designed to be used as a control is experiments such as Southern blot hybridizations or PCRs. It has nonetheless been successfully used as an array CGH control but sequencing a new batch of this sample would have a very limited comparability. Unfortunately, the correlation of this in silico log2 ratios with the CGH log2 ratios was extremely poor (Fig. 46), independently of windows being control regions in one tissue, both or none. We repeated the analysis by using ranks, to avoid incorporating noise, but still no correlation was found.

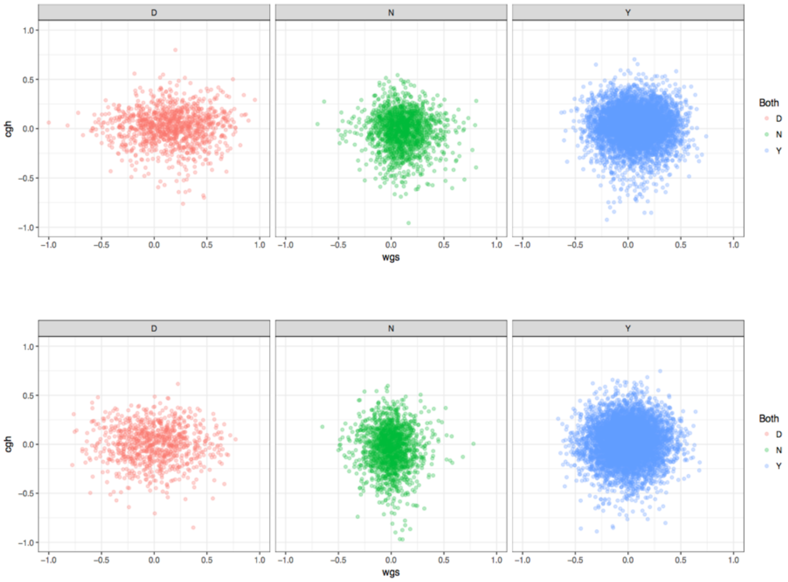


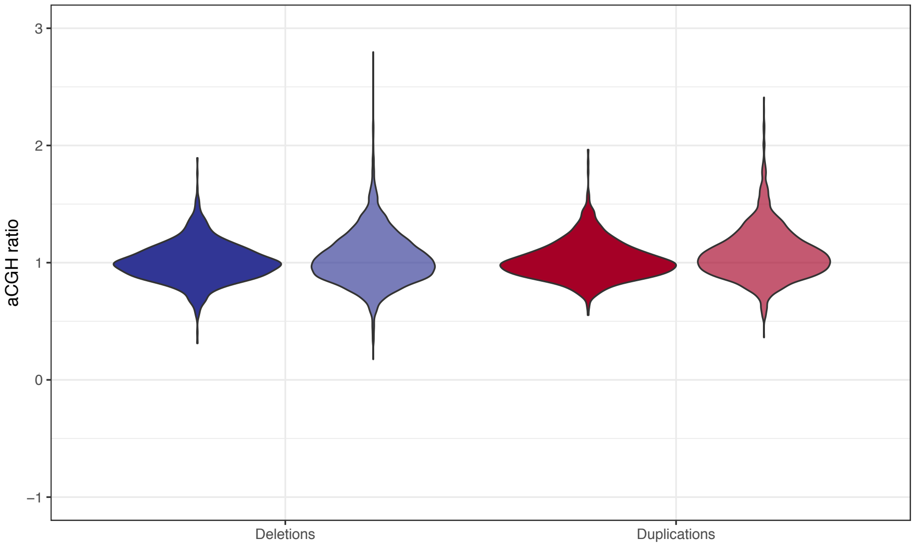
Figure 46. aCGH log2 ratios vs in silico WGS log2 ratios. Colors correspond to windows where both tissues are control regions (blue), only one is (pink) or none (green).

Comparison of copy number calling with CNVnator

Since the results were so poor, CNVs were also called with CNVnator. A window length of 100 bp was used, as recommended for the mean coverage. Then, calls were filtered so that the t-test p-value or the gaussian p-value were < 0.05 and the proportion of reads with q0 was smaller than 0.5. The mean coverage in each of the regions passing this filter was calculated. The mean Pearson correlation with the normalized RD was 0.82. A contingency table showed that the calls were CNVnator and the mean coverage were pointing towards opposite events had one of the p-values > 0.05. Hence, calls were filtered so that both p-values were smaller than 0.05, resulting in a mean correlation of 0.95 and 98.3% of calls agreeing with mean CN. The mismatches were deletion calls in CNVnator with CN 2 according to the mean coverage, showing overall good quality filtered calls.

Events present in multiple individuals in the WGS data are more probable to be also present in the Promega control genome, as they are expected to have higher population frequency, so we removed this variants. Since somatic copy number variants are difficult to call with a caller such as CNVnator, we started by taking calls overlapping in both tissues of each individual. Surprisingly, mean coverage per region had a mean Pearson correlation of 0.14. To check if one class of copy number variant was driving the poor correlation, they were stratified by type. Also, if the reason of the poor correlation was that we were considering events that were also present in the control sample, we reasoned its comparison with the other samples whose whole genome we sequenced would show the opposite pattern, so we checked the aCGH log2 ratio distribution in the same sample were calls were made as well as in the other WGS samples (Fig.47). Still, the distributions were all centered on 1, demonstrating contradictory results. More, the segmented log2 ratios presented a comparable patter (Fig. S17)

Figure 47. aCGH normalized log2 ratio distribution at WGS calls. Dark colors indicate log2 ratios are from the sample were the WGS call was made, whereas lighter colors show the log2 ratios of the other sequenced samples at those same calls.



Somatic mutations in a neurotypical individual

1. The Brain Somatic Mosaicism Consortium

Rationale

The Brain Somatic Mosaicism Network is an American consortium whose objective is to study somatic mosaicism in human brains. The project is focused on understanding the role of somatic mosaicism in neuronal diversity within neurotypical individuals and their relevance in complex neuropsychiatric disorders.

The different 18 research groups participating are focused on particular diseases: autism spectrum disorder, schizophrenia, Tourette syndrome, bipolar disorder, or epilepsy. Results from the study of somatic mutations in these disorders may lead to the discovery of biomarkers and genetic targets to improve the treatment of neuropsychiatric disease and may offer hope for improving the lives of patients and their families (McConnell et al. 2017). To this end, a resource of deep whole genome, whole exome and single-cell sequencing is being generated from patients of the different diseases.

Phase I

Due to the lack of well-established pipelines to call somatic mutations, specially from WGS data, the first phase of the consortium has consisted on identifying variants in a neurotypical individual, also termed *the common experiment*. Each group called somatic variants from the same tissues so that the comparison and validation of the results could help in stablishing somatic calling best practices (unpublished).

Brain and fibroblast samples were obtained from the autopsy of a 55-year-old male. DNA was extracted from a prefrontal cortex (PFC) sample and aliquots were sequenced by several groups, producing technical replicates with a mean coverage of 200x and 90x (table 9) as well as other lower coverage whole genome sequencing data or exome sequencing data. A distinct PFC sample from the same individual was obtained by our group. DNA was extracted in the ancient DNA laboratory and it was sequenced at 90x, generating a biological replicate. Skin fibroblasts were expanded in culture and two deep WGS replicates were also generated.

Table 9. Sample ID and coverage. Replicates of deep whole genome sequencing from the same individual. Depth of coverage after removing duplicates is shown.

Sample ID	Tissue	Coverage
B04	PFC	76x
B03	PFC	86x
F03	Fibroblasts	70x
B06	PFC	204x
F06	Fibroblasts	234x

2. Calling somatic variants from deep WGS

We called somatic variants in the higher sequencing depth samples (table 9). To do this, a similar strategy to that used in the first section of the results was applied. Variants were called for each sample with HaplotypeCaller -ploidy 10. They were then annotated with our custom python script in order to incorporate the information needed for detecting biases. In-depth explanations on each of the noise sources can be found at 3.1 in the Somatic mutations in Parkinson section. Briefly, we required variants at each sample to comply with the following filters:

1. Not multiallelic
2. Not overlapping somatic non-callable tracks (1000G strict mask, segmental duplications and mappability for the 150mers)
3. Not overlapping CNV calls (CNVnator)
4. No more than 3 variants close by
5. Not within 5 bp of indels
6. Not by homopolymers
7. AD >=5
8. R1 and R2 ratio between 0.5 and 2 for each allele
9. Non-significant FET for strand and allele
10. Non-significant Poisson for strand
11. Significant binomial VAF test (<0.01)
12. At most 3 haplotypes
13. Unbiased position in reads for both alleles
14. Not present in gnomAD

Of note, since all replicates come from the same sample, a set of different individuals would be required to help us to identify systematic sequencing errors. Depending on the coverage and allele balance cut-offs used to call variants in human population studies, a portion of this variants can appear as low population frequency polymorphisms. For this reason, we compared the number of variants passing filters 1 to 13 to the number of variants also not present in the gnomAD database (table 10).

As previously discussed, differentiating heterozygous and somatic mutations can be difficult. With the exomes we used the multiple tissues per patient to help us to identify heterozygous variants. Here we can also use the replicates to increase our power. Nonetheless, these deep WGS samples are very expensive, making it interesting to compare the results we would get without the use of replicates, a more realistic scenario. Variants present in population databases can also be useful to identify heterozygous mutations.

The number of variants obtained after applying any filter tackling heterozygous mutations is one order of magnitude higher in both fibroblast samples. This indicates that many mutations that appeared during clonal expansion in their culture are confounded with somatic mutations. This is especially patent from the huge difference in the proportion of calls retained after filters 1-13 that are heterozygous in the brain depending on the tissue they were called at (table 10). We decided to assess the probability of a variant coming from the germline only from brain replicates. This is because we detected a few variants passing all filters in the three brain replicates with a non-significant binomial test in at least one of the fibroblast samples.

After each group made their callings, a set of the candidate variants was selected for validation. Amplicon sequencing was performed by our collaborators in DNA from the same brain as well as NA12878 to serve as a control for systematic sequencing errors. 10X linked reads sequencing was obtained by some groups, and the phasing of candidate positions with both germline haplotypes or with more than 90% of the reads from one of them was considered as an indication of noise and heterozygous variants, respectively. Single cell sequencing of neurons and glia coming from the same PFC were also genotyped. They were especially useful for identifying biased sequencing errors, since they appear at very low frequencies in most if not all the cells (unpublished data). We used these results to evaluate the specificity of our approach (table 10). As we do not know the true set of variants, sensitivity cannot be determined. However, because variants were prioritized for validation with this orthogonal data, our validation rates are inflated by ascertainment bias, very obvious in our calls from F06.

Table 10. Number of variants passing filters and validation rate. The total number of variants passing filters from 1 to 13 or adding the gnomAD filter are shown. Variants were further filtered

		B04	B03	F03	B06	F06
Filters 1-13	Total number	5466	5142	5531	6239	8062
	Not heterozygous in brain	123	105	894	183	2006
	Validation	11/15	13/15	4/5	21/21	3/3
Filters 1-13 + not in gnomAD	Total number	66	56	733	71	1720
	Not heterozygous in brain	44	30	707	44	1688
	Validation	9/12	11/12	4/4	19/20	3/3

Interestingly, even if the validation rate is higher than 75% for each sample, the overlap between the variants discovered in brain replicates, that passed the 14 filters and were not heterozygous in other replicates was low (Fig. 48). The nine variants shared by more than one replicate were all validated successfully. Variants validated but not recovered from every sample were mostly due to absence of call by HaplotypeCaller due to low alternative allele supporting reads or because they did not reach the minimum of 5 we required.

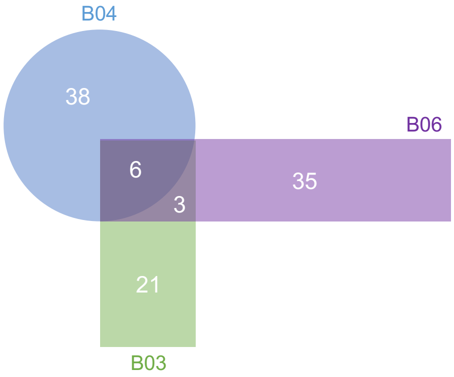


Figure 48. Intersection diagram of brain replicates filtered calls. Overlap between variants called in the brain replicates, passing all the filters, including absence from gnomAD and with significant binomial test in the other two brain replicates.

3. Using deep WGS replicates to identify germline variants

Variant allele frequency at high-confidence heterozygous positions, defined as those present in the common dbSNP database and with a variant allele frequency between 0.4 and 0.6 in at least one sample, showed high dependence on the coverage (Fig. 49). The replicates with coverage higher than 200x have a narrower VAF dispersion than a theoretical binomial distribution, showing the remarkable dependency on coverage for establishing frequency thresholds for separating germline from somatic variants. Nonetheless, outliers exist far from the expected range, even below VAFs of 0.2. Inspection of these variants showed that their frequency was affected by the clonal expansion of fibroblasts. They were either somatic mutations whose frequency increased on culture or heterozygous mutations that decreased in frequency.

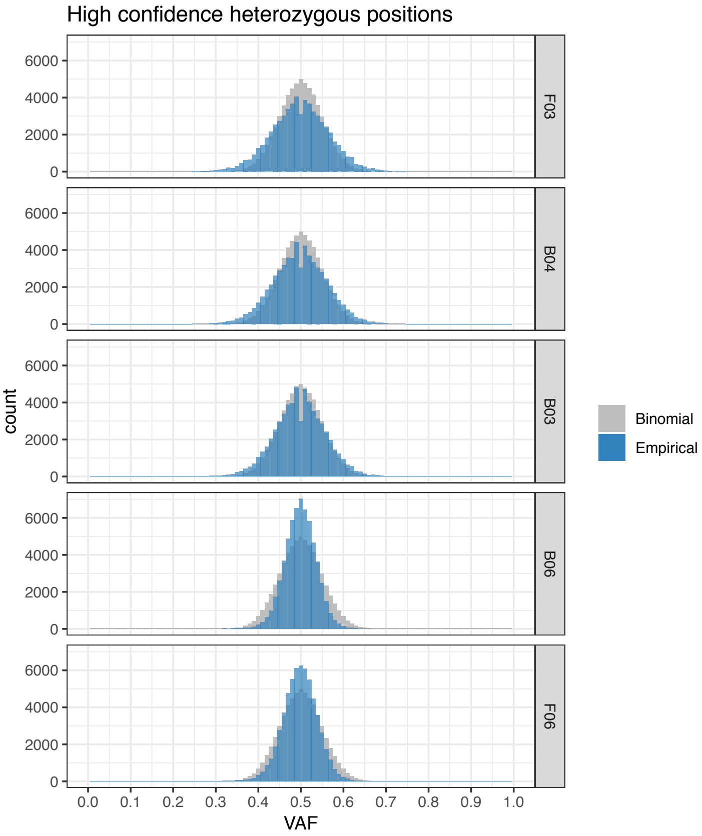


Figure 49. Variant allele frequency dispersion is smaller at higher depths. Histogram of VAFs at high-confidence heterozygous positions (blue) vs a random binomial distribution with p=0.5 (grey). Each panel shows the VAF distribution for one of the replicates, ordered by depth. High-confidence heterozygous positions are those present in the common dbSNP database and with at least one tissue having a VAF between 0.4 and 0.6.

The high depth of coverage of these samples gave us much more power to determine heterozygous positions. Nonetheless, by using only one sample to perform a binomial test on the read counts ~3% of the high-confidence heterozygous variants would be rejected and considered as somatic (Fig. 50). Using two replicates we would be able to correctly classify more than 99.5% of the variants, even when using the expanded fibroblasts or the two lower brain samples.

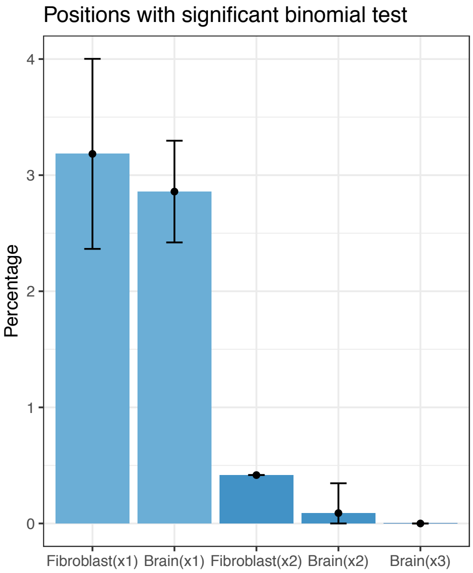


Figure 50. Proportion of high-confidence heterozygous variants misidentified as somatic. Median percentages of high-confidence heterozygous positions that would be classified as not heterozygous because their binomial test in the indicated samples is significant (p-value<0.05). Black bars show they standard deviation. For each number of replicates, all possible comparisons were performed.

DISCUSSION

The interest of somatic mutations in neurodegenerative diseases

The number of studies demonstrating that somatic mutations can cause diseases has been increasing for the past few years (Gleeson et al. 2000; Messiaen et al. 2011; Poduri et al. 2012; Priest et al. 2016; Bar et al. 2017; Dou et al. 2017; Park et al. 2018; Nicolas et al. 2018; Mensa-Vilaró et al. 2019), spanning from cardiac arrhythmia to autism spectrum disorder. Even if the importance of a small proportion of cells carrying a deleterious mutation can seem questionable, these studies show that low frequency variants, even in as little as 0.8% of the DNA, can produce harmful phenotypes. In the brain, the organ of interest for this thesis, a small number of mutated neurons are sufficient to impair neural function, as shown by studies on focal dysplasia patients (Lim et al. 2015, Lim et al 2017). Whether the same holds true for neurodegenerative diseases is an open question worth exploring.

Associating genetic causes to disease when the variants are not inherited can be counterintuitive. However, multiple diseases are caused by recurrent mutations in the same genomic locations. Point mutations reoccur in genes with CpG rich regions (Agarwal et al. 1998) where spontaneous deamination is frequent; in short tandem repeats slippage occurs (MacDonald et al. 1993) and regions flanked by segmental duplications are repeatedly duplicated (Inoue et al. 2001), all causing disease. In a similar way, it is reasonable to assume that the processes involved in the appearance of somatic mutations will make specific genomic regions more susceptible to their appearance. In fact, recent evolutionary divergence has been shown to be variable along the human genome and to correlate with replication timing (Stamatoyannopoulos et al. 2009). In cancer samples, where mutation is so frequent that these patterns become more apparent and faulty repair mechanisms can be used to infer their role, it has been demonstrated that multiple genomic features such as GC content, transcription (Hodgkinson, Chen, and Eyre-Walker 2012) and euchromatin (Schuster-Böckler and Lehner 2012) correlate with the mutation rate and that differential mismatch repair underlies this variation (Supek and Lehner 2015). Furthermore, the histone modification that recruits this machinery is more abundant in exons than introns (Frigola et al. 2017), explaining, at least partially, their lower mutation rate. The different accessibility of both damage agents and the repair machinery to the nucleotides closer of further away from the nucleosomes at each helix turn further influences these patterns, both in cancer and in the germline (Pich et al. 2018).

The mutation distribution observed in cancer compiles all the possible changes to the genome in a context where mutation control is lost and the only major acting force is positive selection (Martincorena et al. 2017). In contrast, although mutation to the germline seems to follow many of these rules, purifying selection has been observed to influence the events that enter into the population variation pool (Xu et al. 2011; Wang et al. 2016). Non-cancerous somatic tissues can be considered as an intermediate state, because mutations lethal to the embryo appear later in development, and depending on the tissue maintenance mechanism, clones with replication advantage will expand, constituting tumors (Lee-Six, et al. 2018; Moore et al. 2018). Hence, the equilibrium of forces governing somatic mutations in these tissues could vary depending on the developmental stage when they occurred.

Besides these general mechanisms, it has been demonstrated that neuronal activity is mediated by double strand breaks (DSBs) in the promoters of a subset of genes (Madabhushi et al. 2015). Furthermore, DSBs occur during the development of the nervous system, making its repair machinery crucial (Onishi et al. 2017) and suggesting that tissue-specific processes could also induce different types of mutation. It is plausible then that somatic mutations appear in a few brain cells and their interaction, together with predisposing germline variants, cause complex developmental diseases such as autism spectrum disorder (Dou et al. 2017), and through their accumulation over aging produce neurodegenerative diseases. In fact, a decrease in the efficiency of nonhomologous end joining repair with age has been proven both in mouse (Vaidya et al. 2014) and human cell lines (Z. Li et al. 2016), whose restoration suppresses the onset of stress-induced premature cellular senescence, making unrepaired DSBs a potential cause of neurodegenerative diseases.

Identification of somatic mutations in sequencing data: present and future

Identifying single nucleotide somatic variants from sequencing data is still challenging for multiple reasons. Differentiating them from heterozygous germline variants is not trivial. Their frequencies overlap, specially at lower coverages, as we have shown in the exomes dataset by means of comparing the tissues of the same individual. Even at high depth of coverage, such as that of the BSMN samples, their range still overlaps. In both projects we have seen that using replicates or different tissues from the same individual greatly increases the power to discern one from the other. We also observed that the general frequency distribution is shifted towards the reference allele in the exomes, that is, frequencies are generally lower, an effect produced by the capture method. This is not the case in the BSMN samples, whose heterozygous calls are symmetrical around a variant allele frequency of 0.5. Hence, considering whether whole genome sequencing or exome capture were performed, as well as the coverage of the experiment is fundamental for stablishing cut-offs to separate heterozygous and somatic variants when only one sample per individual is available.

We found that many false positive somatic mutations were called in multiple exome samples from different individuals. This is because a considerable proportion of them are a product of systematic sequencing errors, that is, after a certain nucleotide sequence one base is erroneously identified with a given error rate. Because this rate is low, its relevance is minor when identifying germline variants or somatic variants clonally expanded in cancer, especially when a threshold is used to remove this kind of noise. On the contrary, they are highly detrimental for the identification of somatic mutations present in a small proportion of the cells of a single sample.

While the reasons behind these biases are poorly understood (Taub, Corrada Bravo, and Irizarry 2010; Ross et al. 2013), the best way to circumvent them is through the use of control panels. In the Parkinson exomes analyses we used the other individuals as a control panel for the identification of biased sequencing errors. When several individuals seem to carry the same somatic variant, we can infer it is a systematic error. Some of these errors are present in a very high number of samples, clearly indicating they are a product of noise. When a variant is only found in a few other samples it is less clear whether recurrent events could be the cause, since as previously discussed, the rate of somatic mutation is heterogeneous along the genome and especially because we are studying patients of the same disease. However, even if we expect the same molecular routes or the same genes to be affected, the probability of recurrent variants at the same exact position seems to be low (Dou et al. 2017; Mensa-Vilaró et al. 2019) and systematic errors and artefacts are so frequent that until more is known about these events, the simplest approach is to discard all these recurrent calls.

A different method for identifying these errors is through single-cell sequencing. Although whole genome amplification methods can result in quite biased data, with frequent allelic dropout or marked changes in depth along the genome (Borgström et al. 2017), the same technology is used for sequencing the amplified material. Hence, the same biased sequencing errors appear in a small proportion of the single cell reads. Because single cells can only have three defined genotypes in diploid organisms such as humans, this is an unequivocal sign of a systematic error. However, it is still an expensive technique, especially for discovering multiple variants and accurately estimating their frequency in the cell population, so its combination with bulk whole genome sequencing seems to be a better approach at this point.

Unresolved regions of the genome, those not present in the reference, can have a high identity to resolved regions. This is because many of them originate from segmental duplications that arose at some point in the population history (Bovee et al. 2008), but they usually accumulate variants with the passage of time. Reads coming from these reference gaps will map against their paralogous sequence, with a lower efficiency because of differences in their boundaries. This results in regions where a few reads carry a variant, or multiple close variants, and oftentimes reads are clipped where the homology region ends. If the new copy is old enough it can appear in other individuals from the same population and can therefore be identified as an artefact with the use of a control panel, as we saw with the exomes, highlighting the value in using samples from the same population. If duplications are more recent, or even de novo, they can be pinpointed by increased coverage, clipped reads and clustered variants. Nonetheless, somatic copy number variants carrying a point mutation are undistinguishable from somatic point mutations when using short reads.

Some of these noisier regions are implicitly collected by the 1000 Genomes Project strict mask, which was developed from the realization that systematic biases occurred in next-generation sequencing (Gibbs et al. 2015). It is very useful for removing false positive somatic variants (Bae et al. 2018). Masking the reference genome would create coverage depressions around the masked segments, which can lower variant discovering power. Therefore, we used the mask to filter variants after calling. False positive calls accumulate at these regions, but at the cost of it removing a big proportion of the genome – about a fifth of the genome or a tenth of the exome – where no variants can be discovered. The mask was derived from low depth samples, and probably less regions would be affected at higher depths. Since somatic variant discovery requires deeper coverages, the sequencing of panels of samples at high depths can be used to produce a new more exact mask, allowing for the discovery of somatic variants in, hopefully, many of these regions. Alternatively, third generation sequencing technologies, with different error patterns (Laver et al. 2015), could be used as orthogonal methods to interrogate these regions, which as of now, are inaccessible to somatic variant calling from next-generation sequencing data.

In a similar way, the WGAC segmental duplications track (Bailey et al. 2001) defines regions where copy-unspecific mapping errors may arise. However, because non-allelic homologous recombination is much more probable in these regions, a portion of the cells could carry a genuine somatic variant there. Long reads or linked reads could be the solution to differentiate between these scenarios, since they can be used to distinguish between the copies accurately. If a somatic variant is present, a proportion of the long or phased reads will properly span the flanking regions and carry the variant.

Besides technological limitations, study design is also crucial for exploring somatic mutations. Analyses of trios, where both parents and one or several children are sequenced (Rahbari et al. 2016; Jónsson et al. 2017), can be very informative to gain insight on how to distinguish germline from somatic variants and can be used to train calling algorithms. Twin studies where only somatic mutations are different between the individuals can be helpful not only as a ground truth set but also to associate genes or genomic regions to discordant diseases (Vadgama et al. 2019) Finally, mixing experiments are an alternative strategy to formally address the issues we encountered and develop more accurate approaches for somatic variant calling (J. Kim et al. 2019).

Once many variants from different individuals have been identified, we will have more power to expand on the studies analyzing mutational signatures (Rahbari et al. 2016; Bae et al. 2018) which can help us understand how mutations occur during the development of different tissues. Once enough clinical data is obtained, we will be able to explore their relationship with diagnosis, treatment effectiveness or prognosis of different diseases. Since the first cell divisions seem to be especially mutagenic (Yizhak et al. 2019), we could also test whether any controllable factors at fertilization or during pregnancy can affect their burden.

The challenges of studying somatic mutations in neurodegenerative diseases

Neuronal death is the main pathological feature of neurodegenerative diseases. If a somatic mutation is the cause of their death, neurons carrying the variant would be depleted in the tissue remaining at the time of the autopsy. Therefore, the analysis of the extant tissue can be a limited approach to discover the responsible variants. However, this death is selective, that is, it affects only certain cell types. It is then reasonable to believe that this susceptibility depends on features of the affected cells. As an example, the death of dopaminergic neurons in drug induced Parkinson is caused by the high affinity of MPP+ to the dopamine transporter

(Shen et al. 1985). Changes affecting this protein could result in the preferential death of this type of neurons. On the other hand, we know that low frequency somatic variants are present in other tissues even if they come from different germ layers (Lodato et al. 2015). Hence, we can make use of samples from different tissues of the same individual to retrieve the causing variant, as most probably cells in different organs will not be affected by those mutations and remain at the time of the autopsy.

Indeed, most of the somatic mutations we found in the Parkinson patients, 12 out of 16, are exclusive to blood. Thus, the relevance of these variants being related to nervous system functions or even to Parkinson disease genes, as we found, can be questioned. It has to be taken into account that the nervous system and neurodegenerative diseases are studied more than others. Hence, comparing these results to those obtained from a panel of healthy people could help us determine not only if the apparent enrichment on neurological genes is meaningful but also if there is a higher burden of somatic mutations in our patients as it has been found for other complex neurological diseases (E. T. Lim et al. 2017; Dou et al. 2017).

Moreover, it could be argued that it is likely that variants appearing to be blood-exclusive are present in many other tissues in frequencies below our detection limit, which at 60x is higher than 1%, something that could be resolved with amplicon sequencing of the central nervous system samples. Cells carrying these mutations could have been present in a higher proportion of the founder cell population of blood or, more probably, drift could have resulted in higher clonal expansion on this lineage in blood. Determining the dynamics of progenitor cells in development as well as understanding adult tissue maintenance is key to set expectations and to find the anomalies linked to disease, which can in turn point towards therapeutic targets.

The relevance of cell lineage research

The expected scenario in normal tissues is derived from what we know about embryonic development and mutation in the first embryonic divisions. We still have to simplify our calculations, assuming equal contribution from cells to the tissues. However, in the first embryo divisions not all cells contribute to the inner cell mass and the trophoblast, (Kelly, Mulnard, and Graham 1978; Balakier and Pedersen 1982) but the differentiation into epiblast and hypoblast is stochastic (Schrode et al. 2014). Details on the successive cleavages and divisions are not clear, but we have some hints from classic *C. elegans* (Sulston et al. 1983) and modern lineage tracing studies (McKenna et al. 2016) as well as evidence from humans (Lodato et al. 2015) that lineages present before gastrulation contribute to multiple tissues from different germ layers.

Until this gap in our knowledge is closed, we cannot accurately infer the division a variant occurred at just from its frequency, but we still can make an approximation. Since already very early differentiations are known to be stochastic, if later lineage divisions are too, it would only be the first few divisions that do not contribute to the embryo symmetrically. In fact, at the earliest stages cells also contribute to the extra-embryonic tissues, so this could be the sole reason for their unequal contribution to adult tissues (Ju et al. 2017). Nonetheless, with our analyses we are not trying to infer the exact division when a variant occurred, but just to question the logic of it appearing in multiple tissues given its frequency, that is, if it makes sense that a mutation is present where it is given its frequency.

Theories explaining the observed patterns are just beginning to be formulated (Arendt et al. 2016). I would argue that if the first cell divisions are particularly mutagenic, once there are enough cells to enter gastrulation, it would make sense that they are somehow shuffled or mixed, since this would produce an individual whose organs have only a proportion of mutant cells instead of having all the cells in all organs deriving from one germ layer being affected. If the somatic mutation is deleterious, the individual would have a higher fitness in the first scenario, not only because we expect a partially affected organ to function sufficiently but, perhaps more importantly, because their germline would be mosaic, making them able to produce offspring without the mutation. Following this line of thought, since somatic mutations constitute part of the variants being incorporated into the population through the germline, in a similar way that selection has favored recombination in sexual organisms to shuffle variants in the offspring, it is reasonable to think that it would also favor this cell shuffling in the germ cell lineage. Once more is known about cell migration during embryonic development and the responsible mechanisms are discovered, we will be able to test this hypothesis.

The role of non-coding variants in disease

The variants we found in a dataset of Parkinson disease (PD) patients are in many cases missense mutations in genes found to be affected in PD or PD models. The relevance of missense mutations for disease is clear, including Parkinson disease

(Polymeropoulos et al. 1997; Valente et al. 2004). A few others overlap 5′ and 3′ UTR regions instead. The implication of mutations in these regions in disease has also been known for a long time (Chen et al. 2006; Miyamoto et al. 2007). This is because proteins regulating translation bind to them (Wilkie, Dickson, and Gray 2003) and because they are targets of miRNAs (Ørom, Nielsen, and Lund 2008).

The relevance of all types of non-coding sequences is becoming clearer the more we learn about genomic regulation. For many years, loci associated to diseases through GWAs that were not in the exome were inexplicable. After the expansion of epigenomic studies many of them were linked to genes or cell types and this led to more information on the variables and processes involved in certain conditions (Ernst et al. 2011; M. C. King et al. 2012). Nevertheless, many studies are still focused on the exome or even perform target sequencing of a just a few genes. Although this can be useful in monogenic highly penetrant sporadic diseases, this is a limiting approach for complex diseases. It is reasonable to assume that deleterious mutations can be better tolerated in a somatic state, pointing at their putative relevance for understanding complex diseases. Furthermore, depending on the approach used, only a handful of somatic mutations, those that appeared in the first cell divisions, can be discovered. Restricting the analysis to a small region of the genome, specially one less tolerant to change makes their discovery even more challenging. GC content and repair mechanisms are different in the exome than the rest of the genome, so the extrapolation of the observed patterns and conclusions made from this data is restricted. Economic limitations are of course practical limits to whole genome sequencing, more so with high coverage, but it seems probable that the field will move towards it as it becomes cheaper.

Other implications of understanding somatic mutation

Cancer is by far the most studied somatic mutation disease. Once cancer driving mutations occur clonal expansion increases the number of cells, so these mutations are expected to be in the whole population of tumor cells. Variants that appear after this stage can also be recovered by the comparison to the normal tissue, so the impact of improving somatic calling would be small in this context. Nevertheless, the field is moving towards understanding clonal expansion and mutation in healthy tissues , so that the differences between normal cells and those that originate tumors can be found (Martincorena et al. 2015; Martincorena and Campbell 2015; Martincorena et al. 2017). Potentially this will help us to understand the disease and which mechanisms are behind cancer appearance.

On a totally different context, all germline mutations were somatic mutations when they first appeared, so how somatic variants originate, and which factors are involved are fundamental questions for understanding and inferring mutation rates along evolution. The molecular clock assumes that the mutation rate is constant in a lineage. However, it correlates with life history traits such as lower body size and lower generation time (Nikolaev et al. 2007; G. W. C. Thomas et al. 2018). It can be hypothesized that the embryonic developmental stages where fast cell division occurs are mutagenic because the repair machinery has very limited time to act. Since shorter generation time species experience more generations and hence more of such mutagenic divisions in the same period of time, this could explain their higher rates. It has been suggested that the development of the circulatory system can shift the mutational signatures at different developmental stages (Bae et al. 2018). Moreover, it has been proposed that mammalian embryonic development is not properly aligned to other animal groups development, and the fast division periods could happen at different stages (O'Farrell, Stumpff, and Tin Su 2004), explaining more of these differences. A complete understanding of these processes can be used to infer the past events from the sequences of present individuals in population genetics.

CONCLUSIONS

1. Accurate detection of somatic mutations from sequencing data requires careful consideration of the noise sources that can particularly hinder their identification

2. A considerable proportion of sporadic Parkinson disease patients carry somatic single nucleotide variants whose germline mutation has been linked to the disease

3. Array CGH lacks the sensitivity needed to identify somatic copy number variants

4. Similar approaches are efficient to identify somatic mutations in sequencing data with different depths.

5. Sequencing multiple tissues or replicates increases the power to discriminate germline and somatic mutations

METHODS

Somatic mutations in Parkinson disease patients

1. Experimental methods

DNA collection and extraction

Tissue samples from cerebellum, neocortex, striatum and substantia nigra were obtained from the biobank HCB-IDIBAPS. They were collected at autopsies of ten Parkinson's disease patients with a short time between death and time of collection (6 to 18 hours). Blood samples from the same individuals were also obtained. DNA extractions were carried out with the Qiagen DNeasy Blood & Tissue Kit.

Exome sequencing

Library preparation and sequencing were performed by BGI. Genomic DNA samples were randomly fragmented into 150-200 bp fragments. Adapters were ligated and the resulting templates purified by the AgencourtAMPure SPRI beads. Libraries were amplified by ligation-mediated polymerase chain reaction (LM-PCR). The exome was captured with the Exon Focus SureSelect kit from Agilent. Paired-end 100 bp sequencing was performed on an Illumina Hiseq2000 platform.

2. Computational methods

Mapping and processing

The resulting FASTQ files were inspected with FASTQC v0.11.4 (Andrews 2010) and mapped with BWA v0.7.8 *mem* (Heng Li 2013) to the human hs37d5 assembly. Lane-specific read groups were added with Picard Tools v1.95 (Broad Institute 2013). *AddOrReplaceReadGroups* and bams were merged by sample with samtools v1.9 (H. Li et al. 2009). Read duplicates were removed with Picard Tools v1.95 *MarkDuplicates REMOVE_DUPLICATES=true*. Base quality score recalibration and indel realignment were applied following GATK's best practices (DePristo et al. 2011) with GATK v3.6 (McKenna et al. 2010). Secondary alignments were also excluded with samtools *view -F 256* and exome coverage was calculated with BEDTools v2.26 (Quinlan and Hall 2010).

Germline genotyping

Germline variants were called with GATK v3.6. First, GVCFs were obtained for each sample independently with *-T HaplotypeCaller –emitRefConfidence GVCF*. Then, all samples were genotyped together with *-T GenotypeGVCFs* and a standard hard filter was applied with *-T VariantFiltration --filterExpression "QD < 2.0 || FS > 60.0 || MQ < 40.0 || MQRankSum < -12.5 || ReadPosRankSum < -8.0"*.

Principal component analysis (PCA)

A PCA of the hard-filtered genotypes was performed with EIGENSOFT v7.2.1 (Price et al. 2006) and samples were plotted according to the resulting eigenvectors with ggplot2 (Wickham 2009).

Germline annotation

Information on type of variants and gene affected was annotated with snpEff 4.3t (Cingolani, Platts, et al. 2012) *eff.* SnpSift 4.3t (Cingolani, Patel, et al. 2012) *dbnsfp* was used to add population frequencies, effect prediction and conservation information.

Enrichment analysis

Overrepresentation enrichment analysis was performed with WebGestalt (B. Zhang, Kirov, and Snoddy 2005) using the geneontology database (Carbon et al. 2009) for molecular functions and genome protein coding genes as background.

Somatic genotyping

For Varscan 2 (Koboldt et al. 2012) somatic variant calling, mpileup files were obtained with samtools *mpileup* per each individual's group of bams. Then, single nucleotide variants were called with Varscan v2.3.2 *mpileup2snp* with lax parameters: *--min-coverage 1 --min-reads2 1 --p-value 1 --min-var-freq 0.000001 --output-vcf.* Indels were called with *mpileup2indel* with the same parameters.

For HaplotypeCaller somatic variant calling, GVCFs per sample were obtained with GATK *-T HaplotypeCaller -ploidy 10 -A StrandAlleleCountsBySample --*

emitRefConfidence GVCF. Then, all GVCFs were genotyped together per chromosome with *-T GenotypeGVCFs -L chr -ploidy 10 -A StrandAlleleCountsBySample* to obtain somatic SNV and indel calls.

Copy number variant calling

Depth of coverage files were obtained with GATK v3.6 *DepthOfCoverage* and GC content per target was calculated with *GCContentByInterval.* Then, CNVs were called jointly for all samples with XHMM v1.0 (Fromer et al. 2012b) with standard parameters following its recommended best practices.

Short tandem repeats

Short tandem repeats in hs37d5 were determined with Tandem Repeats Finder v.4.09 (Benson 1999) and parameters *2 7 7 80 10 12 500 -h*. Homopolymers were extracted with a custom bash script based on their homogeneous repeat motif.

Reference genome annotation BED files

The 1000G strict mask FASTA files were obtained from the 1000G project FTP (ftp://ftp.1000genomes.ebi.ac.uk/vol1/ftp/release/20130502/supporting/accessible_genome_masks/) and were transformed into a BED file using a custom python3 script. BED files for WGAC segmental duplications, common dbSNP SNPs and mappability for 100mers for hg19 were obtained from the UCSC table browser (Karolchik et al. 2004).

VCF custom annotation and filtering

Both types of VCF files were annotated from BED files with BCFtools (H. Li et al. 2009) and from BAM files with a custom Python script using pysam (Pysam-developers 2009). Variants were explored with IGV (J. T. Robinson et al. 2011) and filtered with custom awk scripts.

Clustering heatmap

The R package *pheatmap* was used to obtain heatmap of the variant allele frequency of somatic mutations at each tissue of the individual carrying the mutation. Tissues were clustered by Pearson correlation.

Exploring somatic copy number variants in Alzheimer disease with array CGH data

1. Experimental methods

DNA collection and extraction

Matched brain and blood samples were obtained from the brain banks Banco de Tejidos CIEN and Banco de Cerebros de la Región de Murcia whose ethical committees approved all protocols. In short, whole brains were obtained by neuropathological autopsy shortly after death. They were then divided into two symmetrical halves through a midsagittal section and cut into coronal, sagittal and transversal slices. Tissue slices were frozen by immersion in isopentane at -50°C and transferred to -80°C for long term storage. Selected sections were immunostained to confirm the diagnosis. Classification and staging of Alzheimer were performed according to the CERAD criteria as well as Braak staging of neurofibrillary pathology. The hippocampus regions CA1-CA3 were later dissected from the frozen slices by means of a stereomicroscope.

Samples for total of 26 individuals, including 21 Alzheimer's disease (AD) patients, 2 vascular dementia (VaD) patients and 3 controls were obtained. Additionally, 3 cerebellum samples were obtained from the dissections for 3 of the AD patients. DNA extractions were performed with the Qiagen DNeasy Blood & Tissue Kit.

Comparative genomic hybridisation (CGH) array

The resulting 55 DNA samples were hybridized in two different batches to the NimbleGen Human CGH 3x720K Whole Genome Exon-Focused Array CGH from Roche Diagnostics together with the commercially available control genome Male Human Genomic DNA from Promega. The test samples were labelled with cyanine 3, a dye that upon excitation with ~500 nm light emits at 532 nm which corresponds to green, whereas the control genome was labelled with cyanine 5, whose excitation wavelength is ~600 nm and emits at 635 nm, which is associated with red.

Whole genome sequencing

Libraries were prepared from the hippocampus and blood DNA samples from five of the AD patients with the TruSeq Nano DNA kit with a fragment size. Then they were sequenced in a HiSeqX machine at a mean coverage of 20x.

2. Computational methods

Normalization

Raw light intensities for both test and control emission colors at each probe from each batch were normalized by quantile normalization with nor*malize.quantiles* from the preprocessCore R package (Bolstad 2016). Then, log2 ratios between test and control intensities were taken.

Copy number segmentation and calling

Normalized log2 ratios were segmented with *segmentData* from the R package CGHcall (van de Wiel et al. 2007) with parameters *method="DNAcopy", undo.splits="sdundo", undo.SD=3*. Copy numbers were then called with *CGHcall nclass=5* and *ExpandCGHcall* was used to get the final object.

Whole genome sequencing processing

FASTQ files were inspected with FASTQC v0.11.4 (Andrews 2010). Reads were trimmed with Trimmomatic 0.36 (Bolger, Lohse, and Usadel 2014) *TruSeq3-PE-2.fa:2:30:10:8:false LEADING:20 TRAILING:20 MAXINFO:131:0.9 MINLEN:36* and mapped with BWA v0.7.8 *mem* (Heng Li 2013) to the human hg19 assembly. Lane-specific read groups were added with Picard Tools v1.95 (Broad Institute 2013). Read duplicates were removed with Picard Tools v1.95 *MarkDuplicates REMOVE_DUPLICATES=true*. Base quality score recalibration and indel realignment were applied following GATK's best practices (DePristo et al. 2011) with GATK v3.6 (McKenna et al. 2010).

Copy number variant calling from whole genome sequencing data

Copy number variants were called with CNVnator 8.17 (Abyzov et al. 2011) with a bin size of 100 bp, as recommended for 20-30x coverage data. Also, mrCanavar 0.51 (Alkan et al. 2009) was used to call copy number variants. First, the human reference hg19 was kmer-masked to avoid getting calls at repetitive regions in the reference. To do this, the assembly was split into 36 bp-long kmers with a sliding window of 5 bp. The resulting sequences were mapped to the same assembly with GEM v2 (Marco-Sola et al. 2012) and kmers mapping more than 20 times were masked from the reference. Then, processed sample reads were split into 75-mers and mapped to the masked reference with GEM. Finally, mrCanavar was used to call copy number variants for each patient's tissue. The same process was followed with 10 Spanish samples from 1000 Genomes Project to serve as control.

Somatic mutations in a neurotypical individual

1. Experimental methods

DNA extraction and sequencing

A prefrontal cortex piece of the *common experiment* brain was obtained. DNA extraction was performed at the ancient DNA laboratory to avoid contamination. All the tools used in the procedure were sterilized under a UV light for at least 30 minutes. The surface of the tissue sample was scrapped off by means of a surgical scalpel while on a container resting on dry ice. Smaller samples were cut from the tissue piece and placed into a 1.5 ml tube. DNeasy Blood & Tissue Kit for DNA extraction was used. A DNA library was prepared with the NebNext Ultra II kit and sequenced on an Illumina HiSeq4000 machine for 2x150 cycles resulting in a 90x mean coverage. Other samples from the sample prefrontal cortex as well as from expanded fibroblasts from the same individual were sequenced by our collaborators (unpublished data).

2. Computational methods

Mapping and processing

FASTQ files from all samples were uniformly mapped with BWA v0.7.16 *mem* (Heng Li 2013) to the human hs37d5 assembly. Read duplicates were removed with Picard Tools v2.12.2 *MarkDuplicates REMOVE_DUPLICATES=true*. Base quality score recalibration and indel realignment were applied following GATK's best practices (DePristo et al. 2011) with GATK v3.7 (McKenna et al. 2010).

Somatic genotyping

The bam from each sample was separated in chromosomes. Samples were then genotyped with *HaplotypeCaller -ploidy 10 -A StrandBiasBySample* from GATK 4.1.2 (McKenna et al. 2010) in 5 Mb windows overlapping 600 bp with the surrounding windows when they belonged to the same chromosome.

Copy number variant calling

Copy number variants were called with CNVnator 8.17 (Abyzov et al. 2011) per chromosome with a bin size of 50 bp.

Reference genome annotation BED files

The 1000G strict mask FASTA files were obtained from the 1000G project FTP (ftp://ftp.1000genomes.ebi.ac.uk/vol1/ftp/release/20130502/supporting/accessible_genome_masks/) and were transformed into a BED file using a custom python3 script. BED files for WGAC segmental duplications and common dbSNP SNPs were obtained from the UCSC table browser (Karolchik et al. 2004). Mappability for 150-mers was calculated with GEM v2 gem-mappability.

Short tandem repeats in hs37d5 were determined with Tandem Repeats Finder v.4.09 (Benson 1999) and parameters *2 7 7 80 10 12 500 -h*. Homopolymers were extracted with a custom bash script based on their homogeneous repeat motif.

VCF custom annotation and filtering

VCF files were annotated from BED files with BCFtools (H. Li et al. 2009) and from BAM files as well as CNVnator output with a custom Python script using pysam (Pysam-developers 2009). Variants were filtered with custom awk scripts.

“The owl of Minerva spreads its wings
only with the falling of the dusk”

G.W.F. Hegel, *Philosophy of Right* (1820)

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SUPPLEMENTARY FIGURES

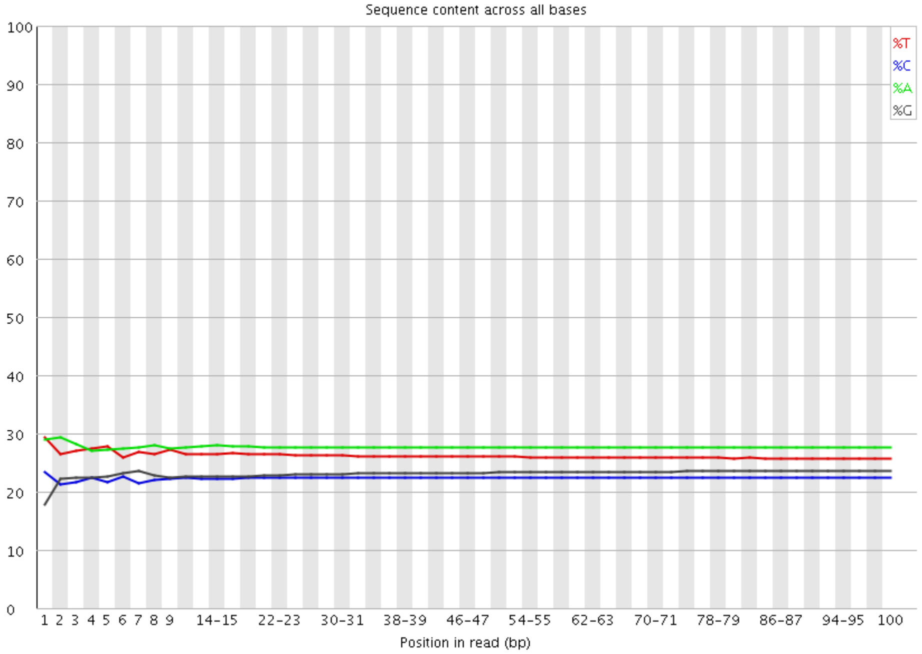

Figure S1. Per base sequence content at DV1C L3 FASTQ. FASTQC output plot for percentage of base pairs carrying each nucleotide (Y axis and the different colours) at each read position (X axis). DV1C L3 is shown as a representative example of every library.

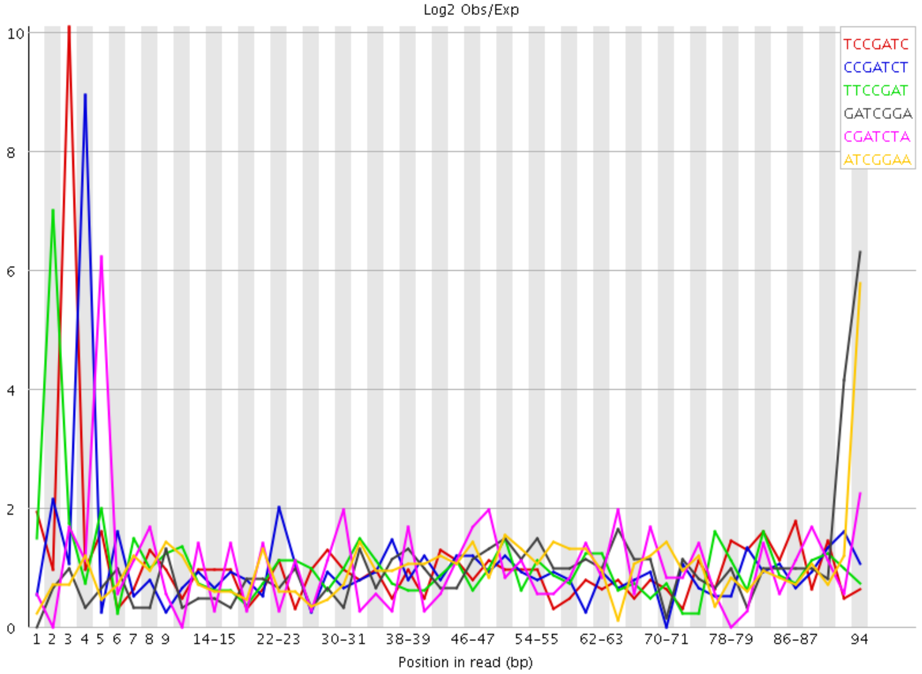

Figure S2. Kmer profiles at DV1C L3 FASTQ. FASTQC output plot for kmer enrichment per read position.

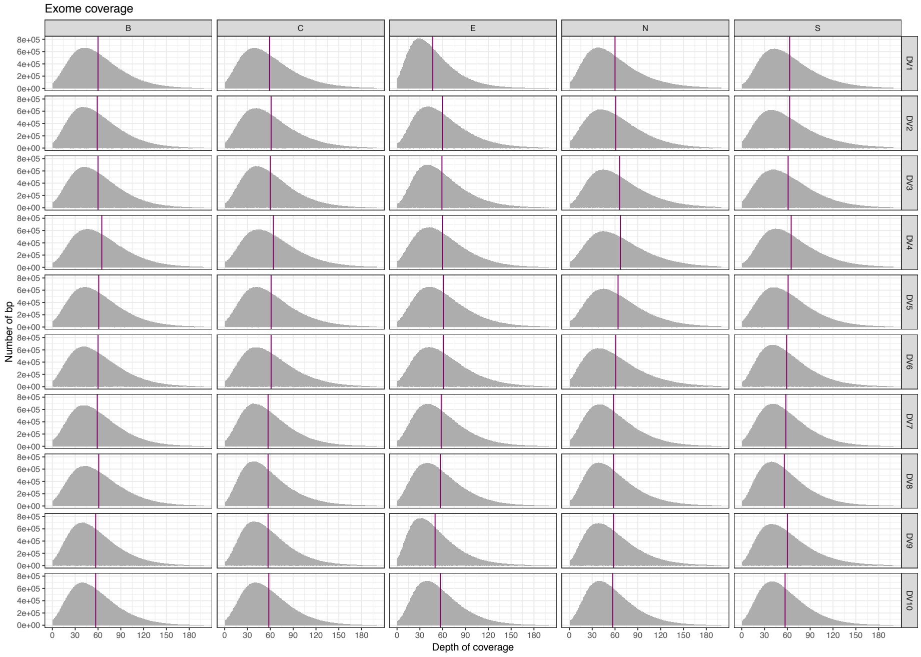

Figure S3. Exome coverage distributions. Histograms showing the number of base pairs of the target region covered by each depth per sample. Tissues are on the X axis facets, blood (B), cerebellum (C), striatum (E), neocortex (N) and substantia nigra (S).

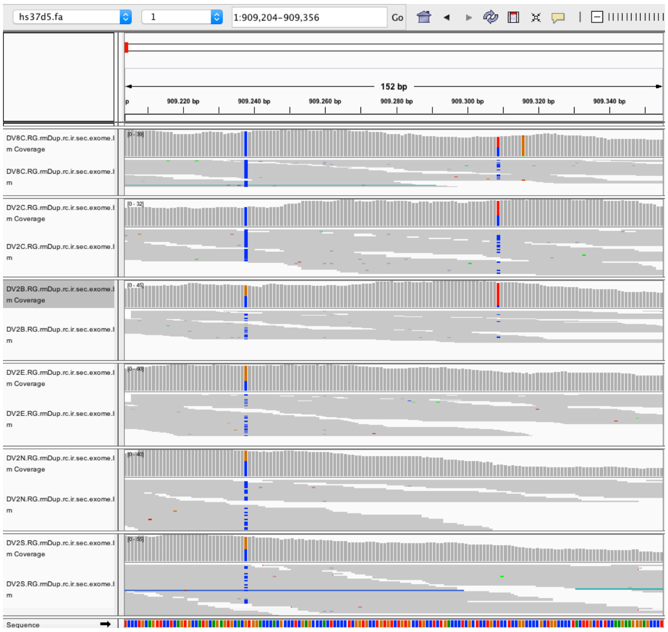
hs37d5.fa
1
1:909,204-909,356
Go
152 bp
909.220 bp
909.240 bp
909.260 bp
909.280 bp
909.300 bp
909.320 bp
909.340 bp
DV8C.RG.rmDup.rc.ir.sec.exome.l m Coverage
DV8C.RG.rmDup.rc.ir.sec.exome.l m
DV2C.RG.rmDup.rc.ir.sec.exome.l m Coverage
DV2C.RG.rmDup.rc.ir.sec.exome.l m
DV2B.RG.rmDup.rc.ir.sec.exome.l m Coverage
DV2B.RG.rmDup.rc.ir.sec.exome.l m
DV2E.RG.rmDup.rc.ir.sec.exome.l m Coverage
DV2E.RG.rmDup.rc.ir.sec.exome.l m
DV2N.RG.rmDup.rc.ir.sec.exome.l m Coverage
DV2N.RG.rmDup.rc.ir.sec.exome.l m
DV2S.RG.rmDup.rc.ir.sec.exome.l m Coverage
DV2S.RG.rmDup.rc.ir.sec.exome.l m
Sequence

Figure S4. DV2C shares variants with DV8C. IGV screenshot showing a region where at one variant, DV8 is homozygous for the alternative allele while DV2 is heterozygous. For the other variant, DV2 is homozygous for the reference allele while DV8 is heterozygous. Vertical bars indicate the coverage per base pair and horizontal grey lines denote reads aligned to the region. Coloured bars indicate variants, with colour depending on the alleles: A (green), C (blue), G (brown) and T (red).

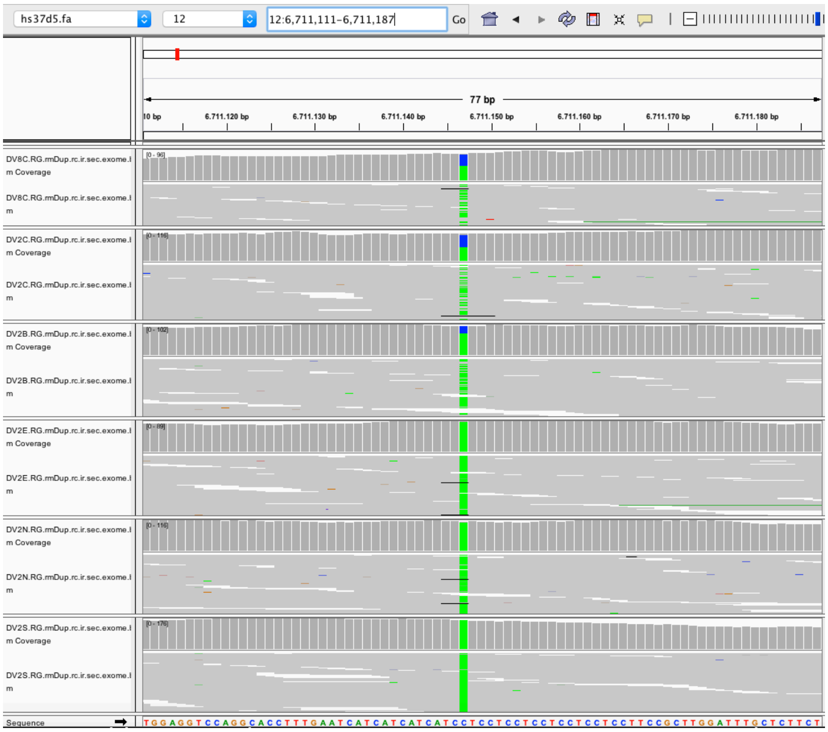
hs37d5.fa
12
12:6,711,111-6,711,187
Go
77 bp
10 bp
6,711,120 bp
6,711,130 bp
6,711,140 bp
6,711,150 bp
6,711,160 bp
6,711,170 bp
6,711,180 bp
DV8C.RG.rmDup.rc.ir.sec.exome.l m Coverage
[0 - 96]
DV8C.RG.rmDup.rc.ir.sec.exome.l m
DV2C.RG.rmDup.rc.ir.sec.exome.l m Coverage
[0 - 116]
DV2C.RG.rmDup.rc.ir.sec.exome.l m
DV2B.RG.rmDup.rc.ir.sec.exome.l m Coverage
[0 - 102]
DV2B.RG.rmDup.rc.ir.sec.exome.l m
DV2E.RG.rmDup.rc.ir.sec.exome.l m Coverage
[0 - 89]
DV2E.RG.rmDup.rc.ir.sec.exome.l m
DV2N.RG.rmDup.rc.ir.sec.exome.l m Coverage
[0 - 116]
DV2N.RG.rmDup.rc.ir.sec.exome.l m
DV2S.RG.rmDup.rc.ir.sec.exome.l m Coverage
[0 - 176]
DV2S.RG.rmDup.rc.ir.sec.exome.l m
Sequence
TGGAGGTCCAGGCACCTTTGAATCATCATCATCATCCTCCTCCTCCTCCTCCTCCTTCCGCTTGGATTTGCTCTTCT

Figure S5. DV2B has an intermediate allele frequency. IGV screenshot showing a region with one variant, where DV8 is heterozygous and DV2 is homozygous for the alternative allele.

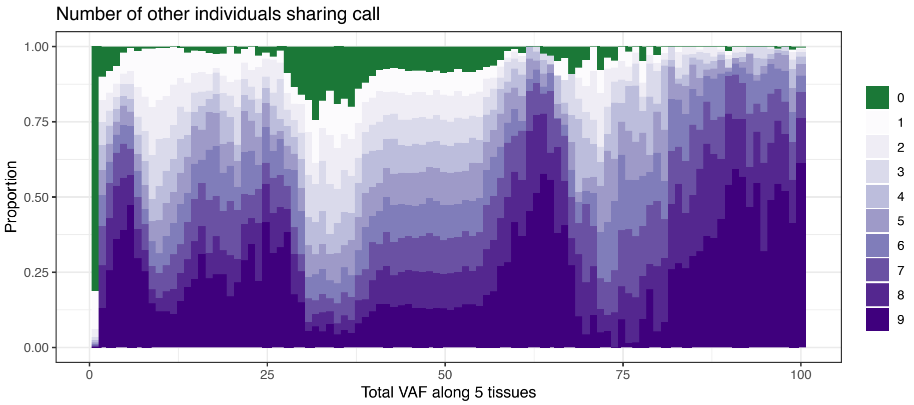

Figure S6. Exclusivity of calls by total variant allele frequency. Each bar shows the proportion of calls with a given variant allele frequency along the 5 tissues of an individual that have been called in none (0), or 1-9 other individuals. Calls were filtered so that their total depth was between 250 and 350.

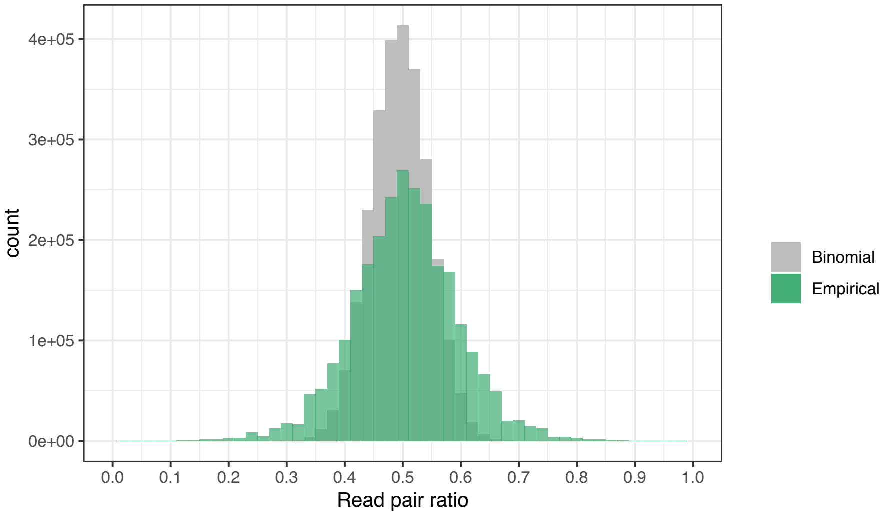

Figure S7. Read pair ratio distribution. R1 depth to R1 + R2 depth ratio at positions with depth from 20 to 100 and alternative depth >4 (green). A binomial distribution with p=0.5 is shown in grey for comparison.

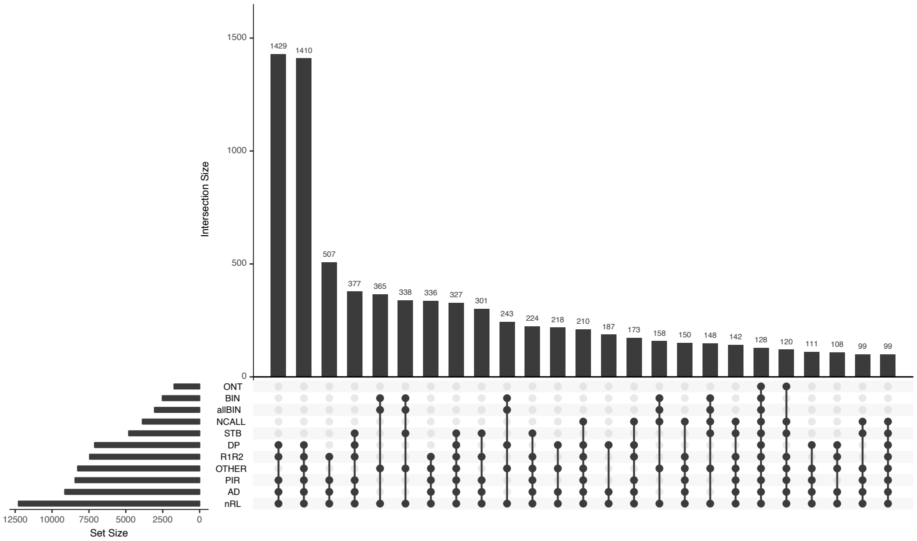

Figure S8. Intersection of calls filtered by number of variants within read length with other filters. Horizontal bars represent the number of variants that do not pass each filter at sample DV1S. Vertical bars correspond to the number of variants in the intersection indicated by the dots. ONT: not on target, BIN: failed binominal test, allBIN: failed binomial test at some tissues, NCALL: non-callable tracks, STB: strand bias, DP: depth <20 or >100, R1R2: read pair bias, OTHER: called in more than 1 other individuals' tissue, PIR: biased position in read, AD: alternative allele depth <5, nRL: more than 3 variants within read length.

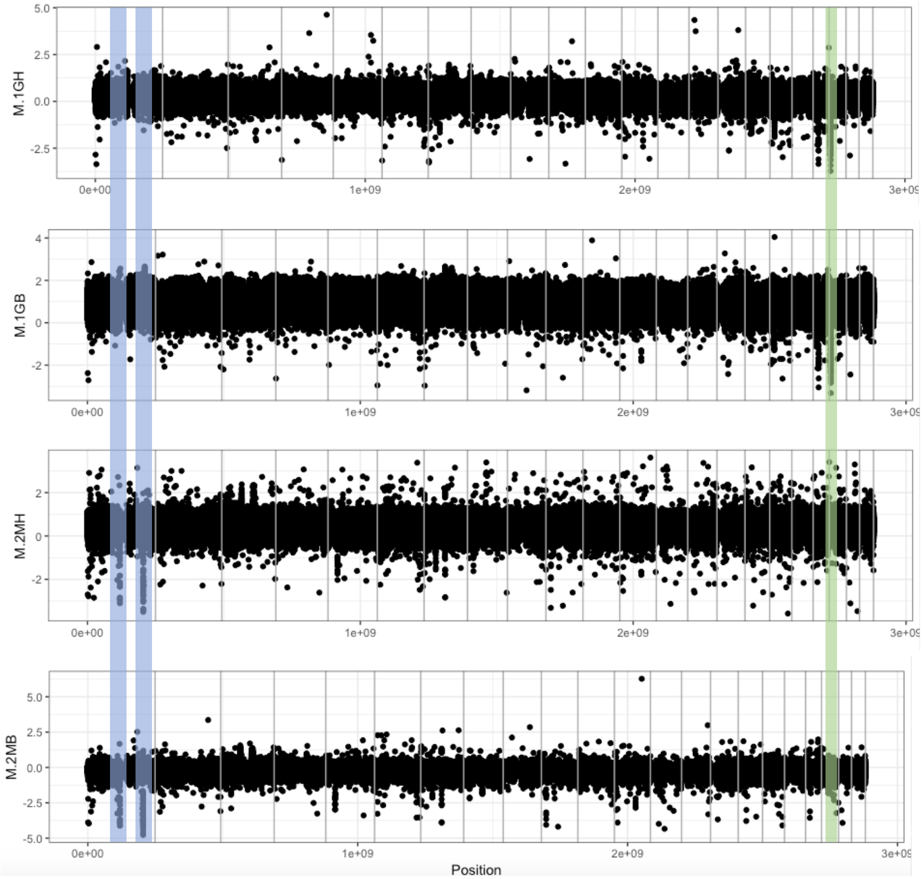

Figure S9. Log2 ratios by position for several samples. Log2 of red/green light intensities per probe (M, in the Y axis) by genomic position (X axis). Blood samples are tagged with "B" and hippocampus samples with "H". Grey lines show limits between chromosomes. Colored bars mark deletions specific of an individual and shared between the tissues, two in individual 2M in blue and one in 1G in green.

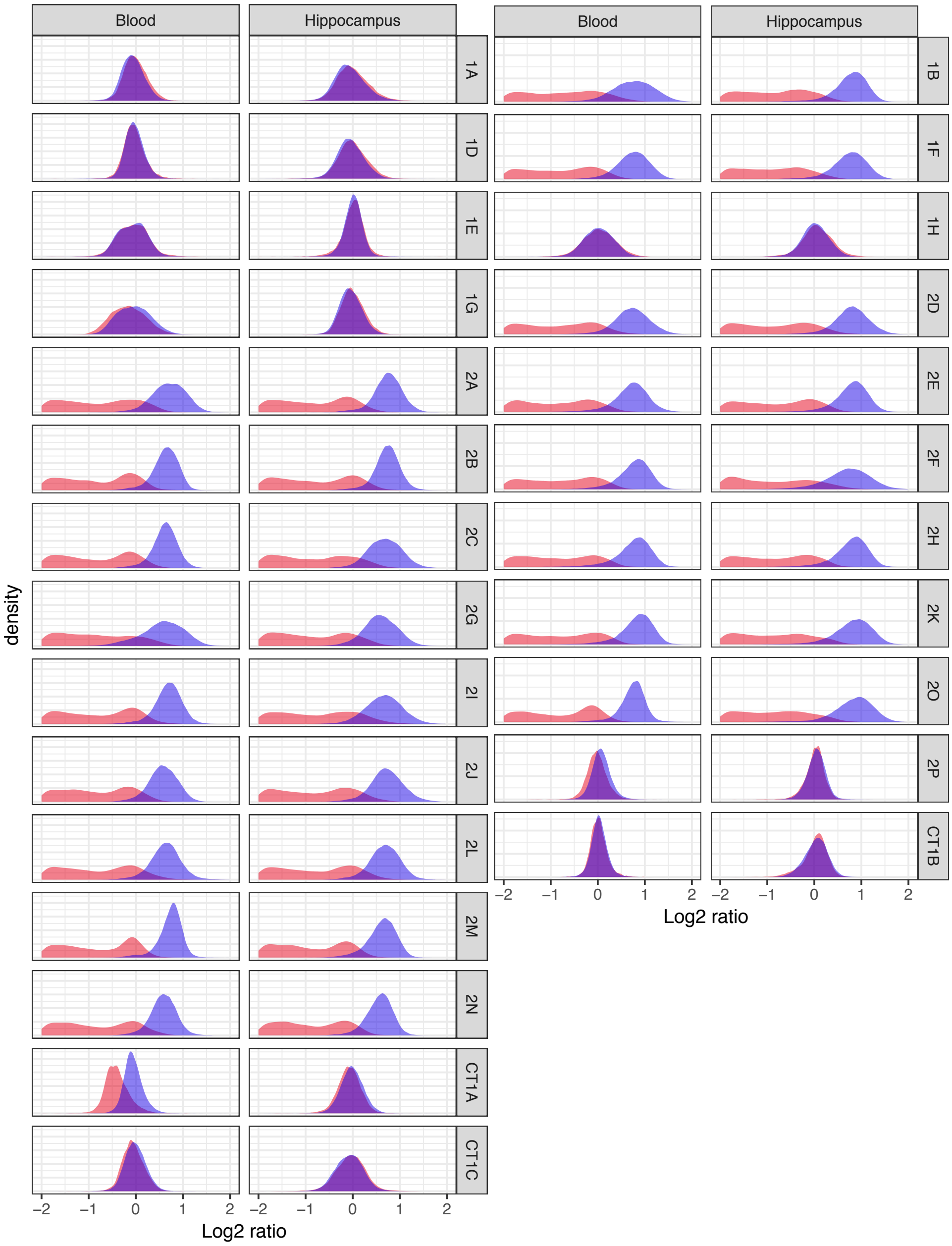

Figure S10. Log2 ratio density at sex chromosomes. Log2 ratios of red to green light intensities at the X chromosome (blue) and the Y chromosome (red) for each individual and tissue.

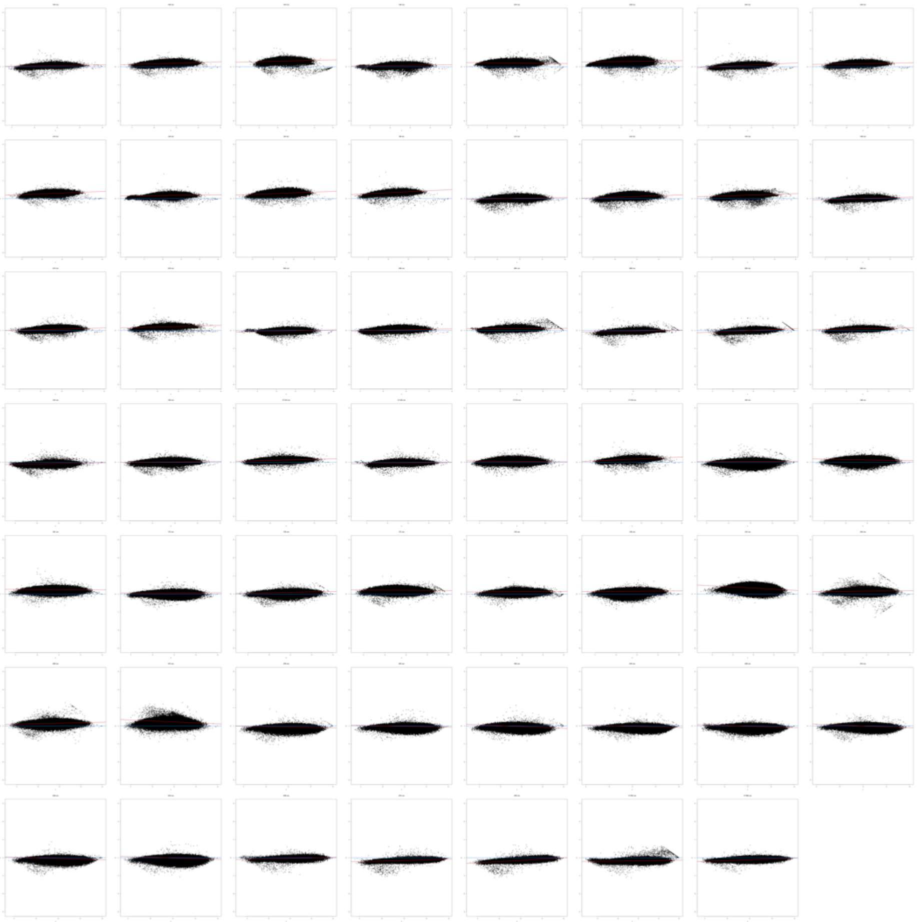

Figure S11. MA plots for all arrays. Mean intensity of both channels (A) against log2 ratio (M) at each probe for each array. Blue lines are at M 0 of no copy number change and red lines are the regression lines.

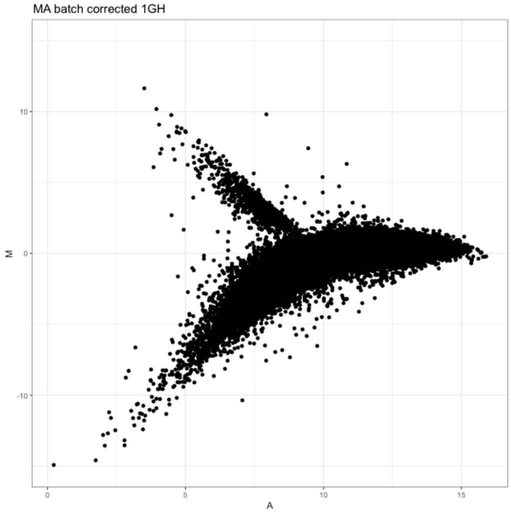

Figure S12. MA plot after batch correction. MA plot of 1GB as an example of the dolphin distribution found after batch correction in some of the arrays.

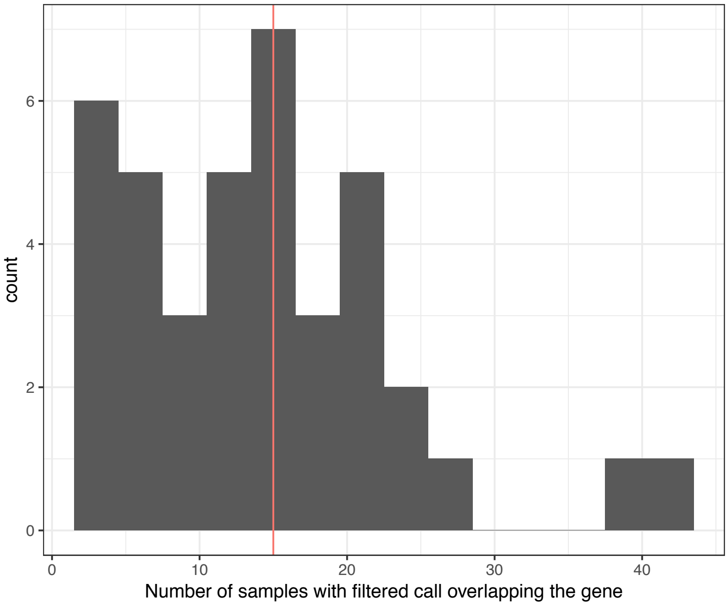

Figure S13. Number of samples called for genes with 360 to 370 filtered probes. The number of samples were *HFE*, with 365 filtered probes, was called at is represented by the red line.

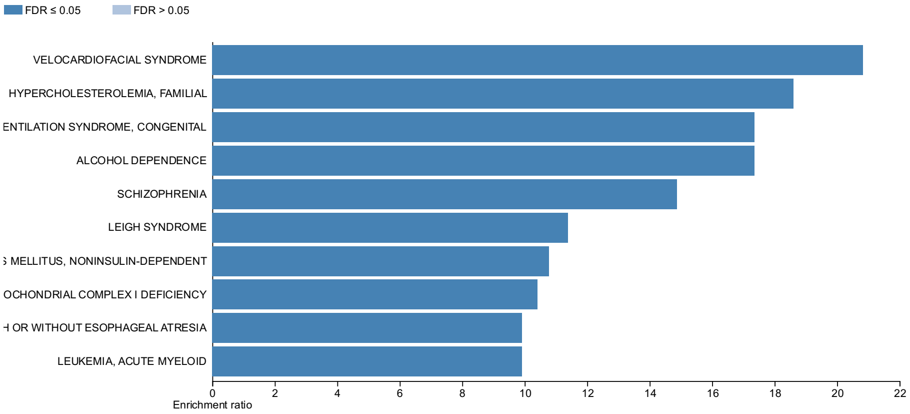

Figure S14. Filtered calls disease enrichment analysis. Overrepresentation enrichment analysis for OMIM genes of all filtered calls in the Alzheimer patients.

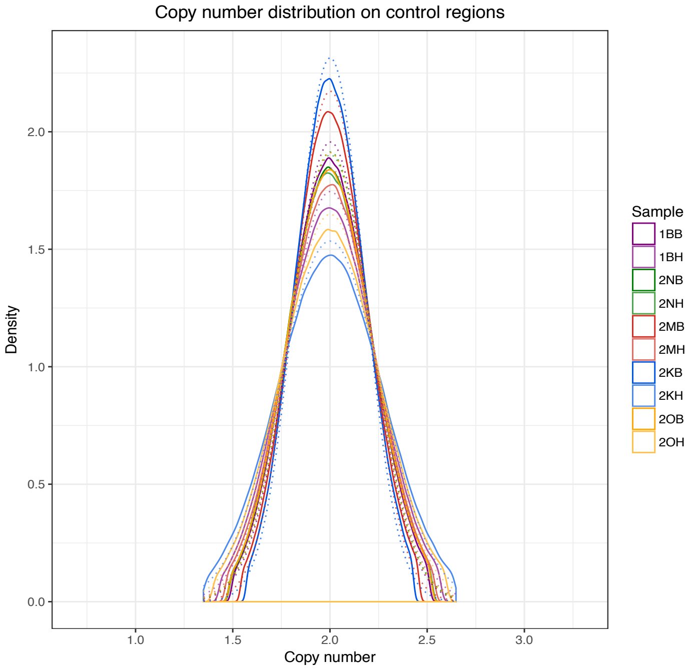

Figure S15. Copy number at control regions. mrCanavar copy number at control regions for each sample (solid lines) compared to a normal distribution with the same mean and standard deviation (dotted lines).

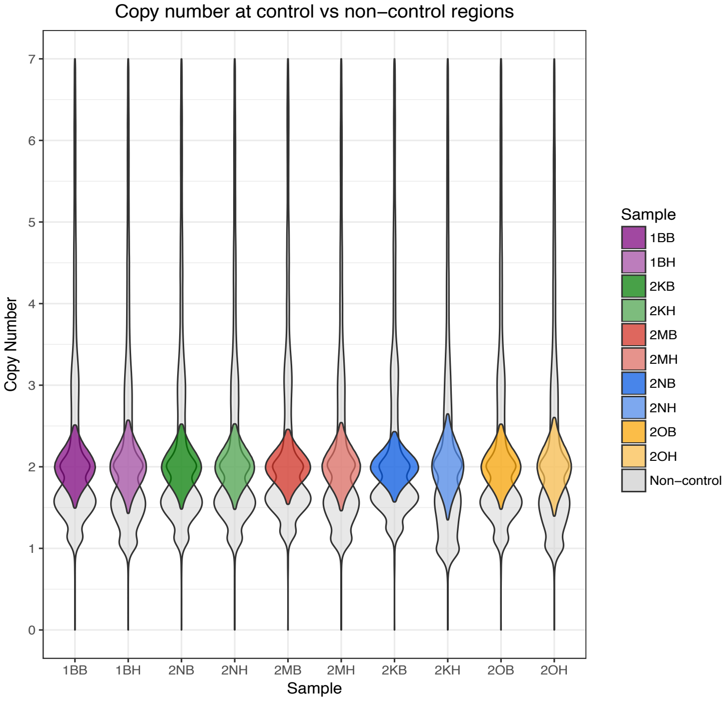

Figure S16. Copy number in and out of control regions. mrCanavar copy number distribution at control regions for each sample (colored violins) and outside control regions (grey violins).

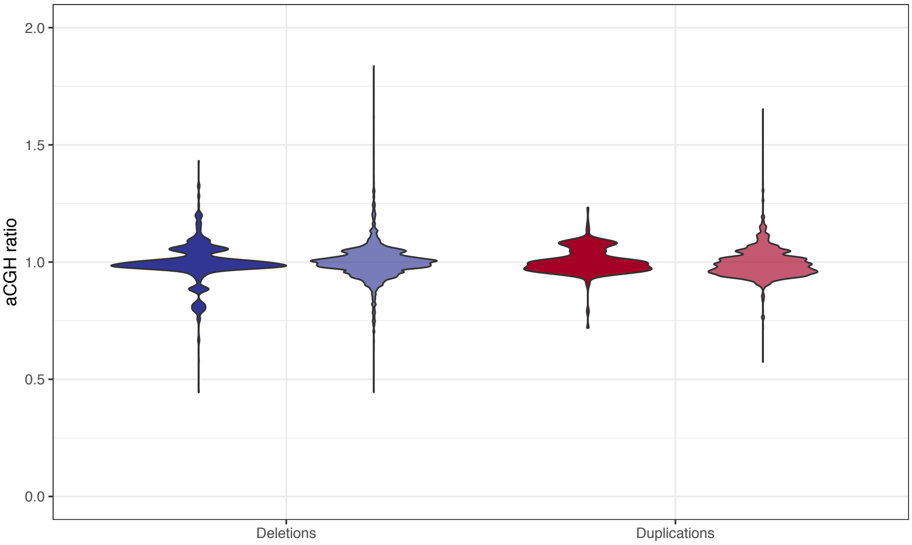

Figure S17. aCGH segmented ratio distribution at WGS calls. Dark colors indicate log2 ratios are from the sample were the WGS call was made, whereas lighter colors show the log2 ratios of the other sequenced samples at those same calls.

SUPPLEMENTARY TABLES

Supplementary table 1. Genes reported at OMIM as involved in Parkinson disease. For each of the loci coordinates in hg19 (same as in hs37d5), the locus and gene names, if appropriate, are reported. Also, the inheritance reported at OMIM: Autosomal dominant (AD), autosomal recessive (AR) or association (AS).

Coordinates in hg19	Locus	Gene name	Inheritance
4:90645250-90759447	*PARK1*	*SNCA*	AD
6:161768590-163148834	*PARK2*	*PRKN*	AR
2:73114512-73119289	*PARK3*	*SPR*	AR
4:90645250-90759447	*PARK4*	*SNCA*	AD
4:41258898-41270446	*PARK5*	*UCHL1*	AD
1:20959948-20978004	*PARK6*	*PINK1*	AR
1:8021714-8045342	*PARK7*	*DJ-1*	AR
12:40618813-40763086	*PARK8*	*LRRK2*	AD
1:17312453-17338423	*PARK9*	*ATP13A2*	AR
-	*PARK10*	-	-
2:233562015-233725289	*PARK11*	*GIGYF2*	AD
-	*PARK12*	-	-
2:74756532-74760683	*PARK13*	*HTRA2*	AD
22:38507502-38577761	*PARK14*	*PLA2G6*	AR
22:32870707-32894818	*PARK15*	*FBXO7*	AR
-	*PARK16*	-	-
16:46693589-46723144	*PARK17*	*VPS35*	AD
3:184032283-184053146	*PARK18*	*EIF4G1*	AD
1:65775218-65881552	*PARK19*	*DNAJC6*	AR
21:34001069-34100351	*PARK20*	*SYNJ1*	AR
3:132136553-132257876	*PARK21*	*DNAJC13*	AD
7:56169266-56174187	*PARK22*	*CHCHD2*	AD
15:62144590-62352664	*PARK23*	*VPS13C*	AR
1:155204239-155214653	-	*GBA*	AS
4:100257649-100273917	-	*ADH1C*	AS
6:170863421-170881958	-	*TBP*	AS
12:111890018-112037480	-	*ATXN2*	AS
13:70681345-70713885	-	*ATXN8OS*	AS
17:43971748-44105699	-	*MAPT*	AS
X:120181462-120183796	-	*GLUD2*	AS
16:89984287-89987385	-	*MC1R*	AS
6:28477797-33448354	-	*HLA*	AS
14:92524896-92572965	-	*ATXN3*	AS

Supplementary table 2. SNPs associated to PD. List of SNPs previously associated to PD in GWAS.

dnSNP ID	hg19 coordinates
rs797906	1:54190695
rs114138760	1:154898185
rs10737170	1:156063880
rs6710823	2:135592381
rs4954218	2:135803425
rs2390669	2:169091942
rs2102808	2:169117025
rs9917256	2:169143035
rs7617877	3:28705764
rs34016896	3:160992864
rs11248051	4:858332
rs6599389	4:939113
rs34884217	4:944210
rs11248060	4:964359
rs1596117	4:77151490
rs356219	4:90637601
rs356220	4:90641340
rs356165	4:90646886
rs2736990	4:90678541
rs13201101	6:32343604
rs3129882	6:32409530
rs1801582	6:161807855
rs12718379	8:16860077
rs1805874	8:91082062
rs2205108	8:91136078
rs7077361	10:15561543
rs10886515	10:121343589
rs1079597	11:113296286
rs1994090	12:40428561
rs1491923	12:40591117
rs1491942	12:40620808
rs11175655	12:40623727
rs34637584	12:40734202
rs10847864	12:123326598
rs4889603	16:30982225
rs12456492	18:40673380
rs4130047	18:40678235
rs117022814	19:2209647
rs7412	19:45412079
rs2823357	21:16914905
rs2010795	21:45172628

Supplementary table 3. Samples hybridized in array CGH.

Codes	Diagnosis	Braak Stage
1G	Alzheimer	II
1A	Alzheimer	III
2G	Alzheimer	III
2A	Alzheimer	IV
2J	Alzheimer	IV
2I	Alzheimer	V
2L	Alzheimer	V
1D	Alzheimer	V
2C	Alzheimer	VI
2N	Alzheimer	VI
2M	Alzheimer	VI
2B	Vascular Dementia	I
1EH	Vascular Dementia	II
CT1A	Control	-
CT1C	Control	-
1B	Alzheimer	III
1F	Alzheimer	V
1H	Alzheimer	IV
2D	Alzheimer	VI
2F	Alzheimer	V
2E	Alzheimer	III
2H	Alzheimer	VI
2K	Alzheimer	IV
2O	Alzheimer	VI
2P	Alzheimer	NA
CT1B	Control	-

Supplementary table 4. Genes reported at OMIM as involved in Alzheimer disease. For each of the loci coordinates in hg19 (same as in hs37d5), the locus and gene names, if appropriate, are reported. Also, the inheritance reported at OMIM: Autosomal dominant (AD), autosomal recessive (AR) or association (AS).

Coordinates in hg19	Locus	Gene name	Inheritance
6:26087509-26095469	6p22.2	HFE	AD
7:150688144-150711687	7q36.1	NOS3	AD
10:75670862-75677258	10q22.2	PLAU	AD
12:9220304-9268558	12p13.31	A2M	AD
17:56347217-56358296	17q22	MPO	AD
21:27252861-27543138	21q21.3	APP	AD

List of publications

Lobon, I., S. Tucci, M. De Manuel, S. Ghirotto, A. Benazzo, J. Prado-Martinez, B. Lorente-Galdos, et al. 2016. “Demographic History of the Genus Pan Inferred from Whole Mitochondrial Genome Reconstructions.” Genome Biology and Evolution 8 (6). doi:10.1093/gbe/evw124.

Olalde, I., H. Schroeder, M. Sandoval-Velasco, L. Vinner, **I. Lobón**, O. Ramirez, S. Civit, et al. 2015. “A Common Genetic Origin for Early Farmers from Mediterranean Cardial and Central European LBK Cultures.” Molecular Biology and Evolution 32 (12). doi:10.1093/molbev/msv181.

Xue, Y., J. Prado-Martinez, P.H. Sudmant, V. Narasimhan, Q. Ayub, M. Szpak, P. Frandsen, Y. Chen, B. Yngvadottir, D. N. Cooper, M. de Manuel, J. Hernandez-Rodriguez, **I. Lobon**, et al. 2015. “Mountain Gorilla Genomes Reveal the Impact of Long-Term Population Decline and Inbreeding.” Science 348 (6231). doi:10.1126/science.aaa3952.