Master 2
MISO/OSB

DNA mutations and prediction of genetic diseases

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UMR 1283
Fonctional (Epi)genomics and Mechanisms of Type 2 Diabetes and Related Diseases

What Are Mutations?

- Changes in the nucleotide sequence of DNA
- May occur in somatic cells (aren't passed to offspring)
- May occur in gametes (eggs & sperm) and be passed to offspring

Are Mutations Helpful or Harmful?

- Mutations happen regularly
- Almost all mutations are neutral
- Chemicals & UV radiation cause mutations
- Many mutations are repaired by enzymes

Are Mutations Helpful or Harmful?

- Some type of skin cancers and leukemia result from somatic mutations
- Some mutations may improve an organism's survival (beneficial)

Types of Mutations

Chromosome Mutations

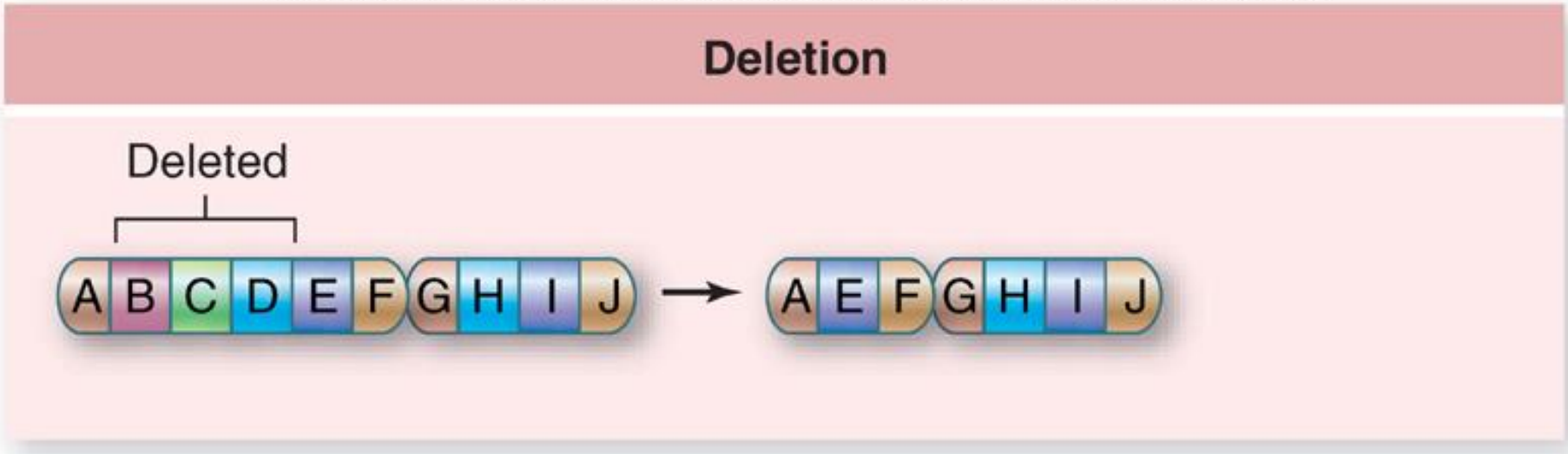
- May Involve:
  - **Change in the structure**
  - **loss or gain**

Chromosome Mutations

- Four types exist:
  - **Deletion**
  - **Inversion**
  - **Translocation**
  - **Nondisjunction**

Deletions

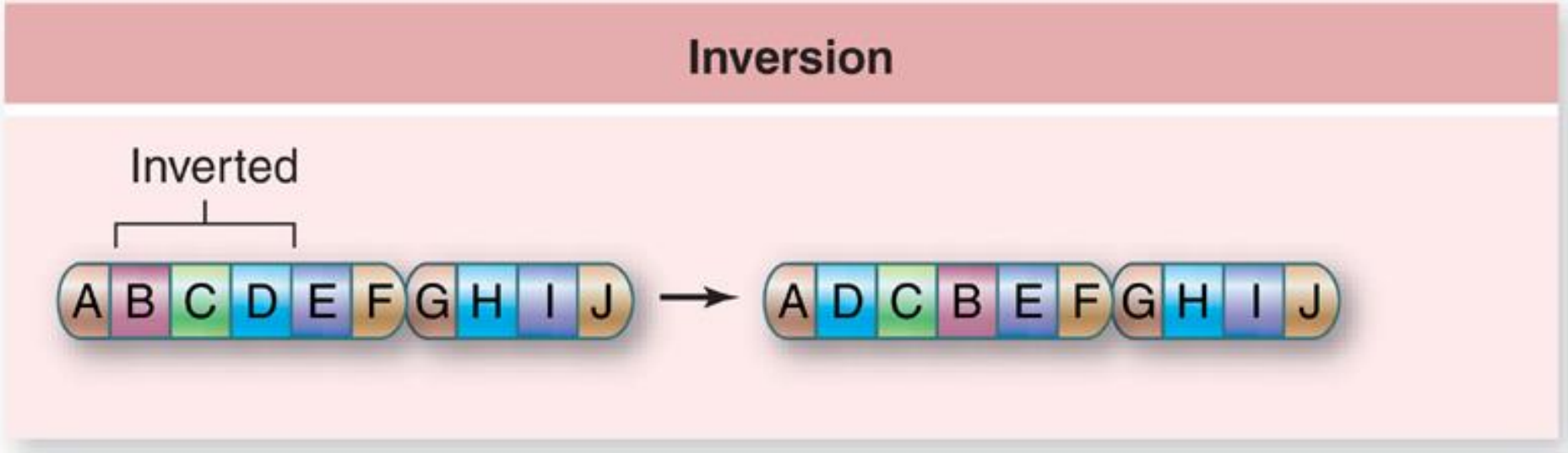
- Due to **breakage**
- A **piece** of a chromosome is **lost**



If too much information is lost, it may be fatal to the organism and may result in early death (e.g., Cri-du-chat syndrome – large deletion from chromosome #5)

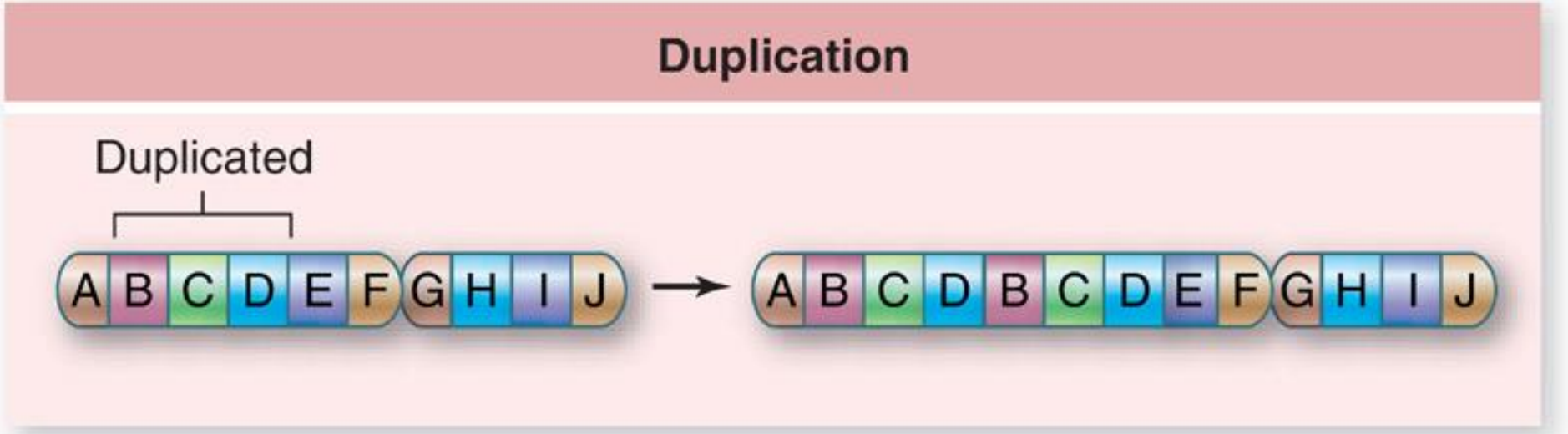
Inversions within chromosome

- Chromosome segment **breaks off**
- Segment flips around **backwards**
- Segment **reattaches**



Duplications within chromosome

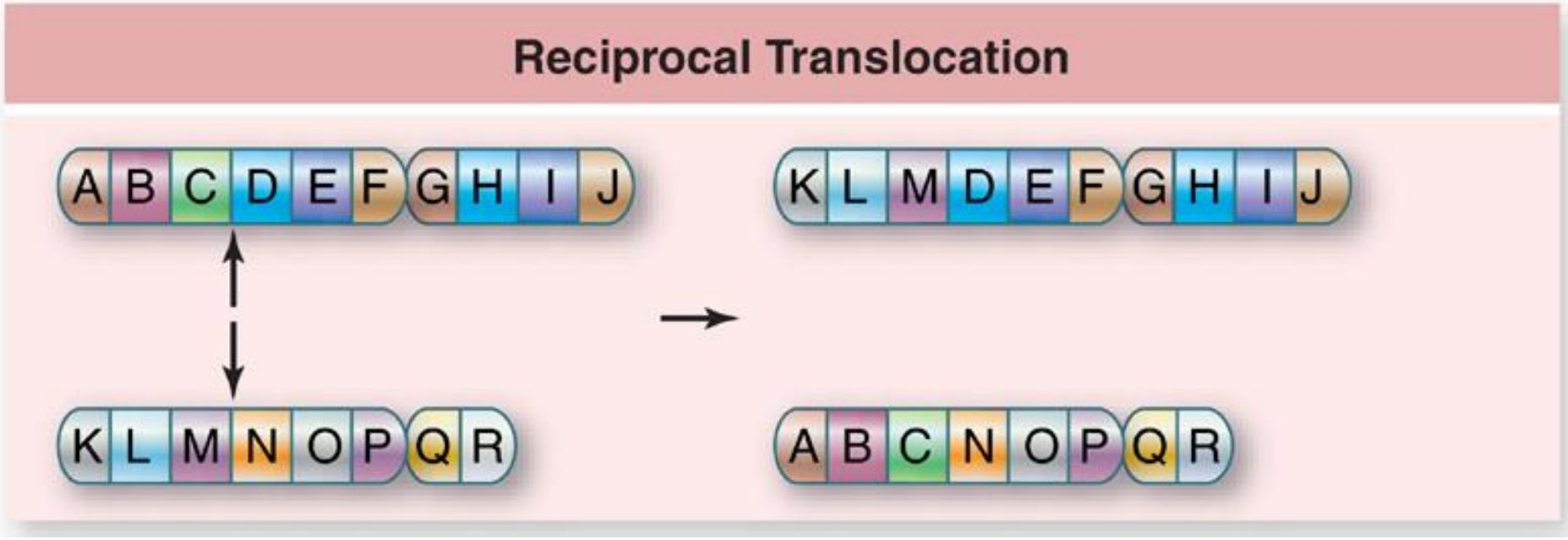
- Occurs when a gene **sequence is repeated**



Effect of base duplications depend on location within the chromosome – whether or not duplication resides in coding or non-coding region of DNA

Translocations within chromosome

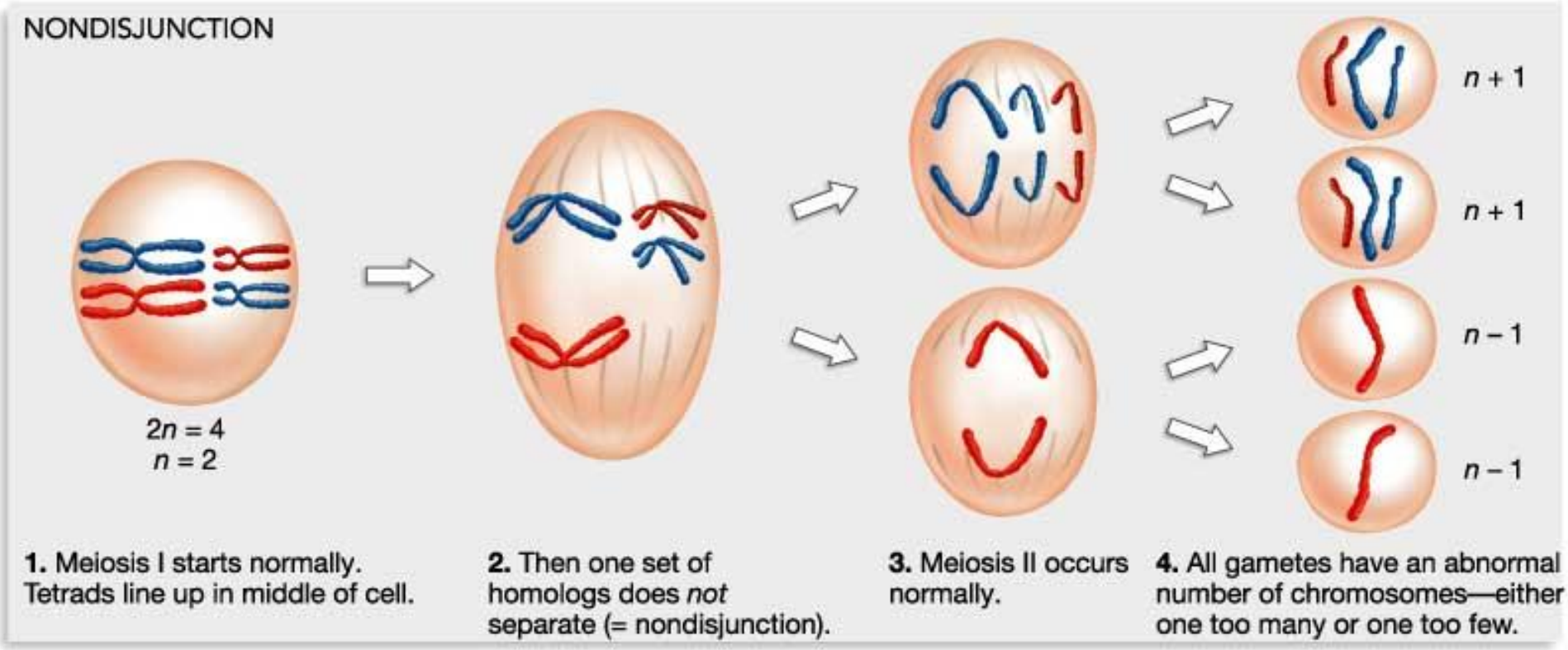
- Involves **two chromosomes** that aren't homologous
- **Part** of one chromosome is **transferred to another** chromosomes



Associated with 2 forms of leukemia – oncogenes translocated to incorrect regions within chromosomes of leukocytes (white blood cells)

Nondisjunction

- Failure of chromosomes to separate during meiosis
- Causes gamete to have too many or too few chromosomes


NONDISJUNCTION
2n = 4
n = 2
n + 1
n + 1
n – 1
n – 1
1. Meiosis I starts normally. Tetrads line up in middle of cell.
2. Then one set of homologs does not separate (= nondisjunction).
3. Meiosis II occurs normally.
4. All gametes have an abnormal number of chromosomes—either one too many or one too few.

⇒ In these disorders, entire chromosomes, or large segments of them, are missing, duplicated, or otherwise altered

⇒ Can be organized in two basic groups:

1/ Numerical abnormalities : when an individual is missing either a chromosome from a pair (monosomy) or has more than two chomosomes of a pair (trisomy)

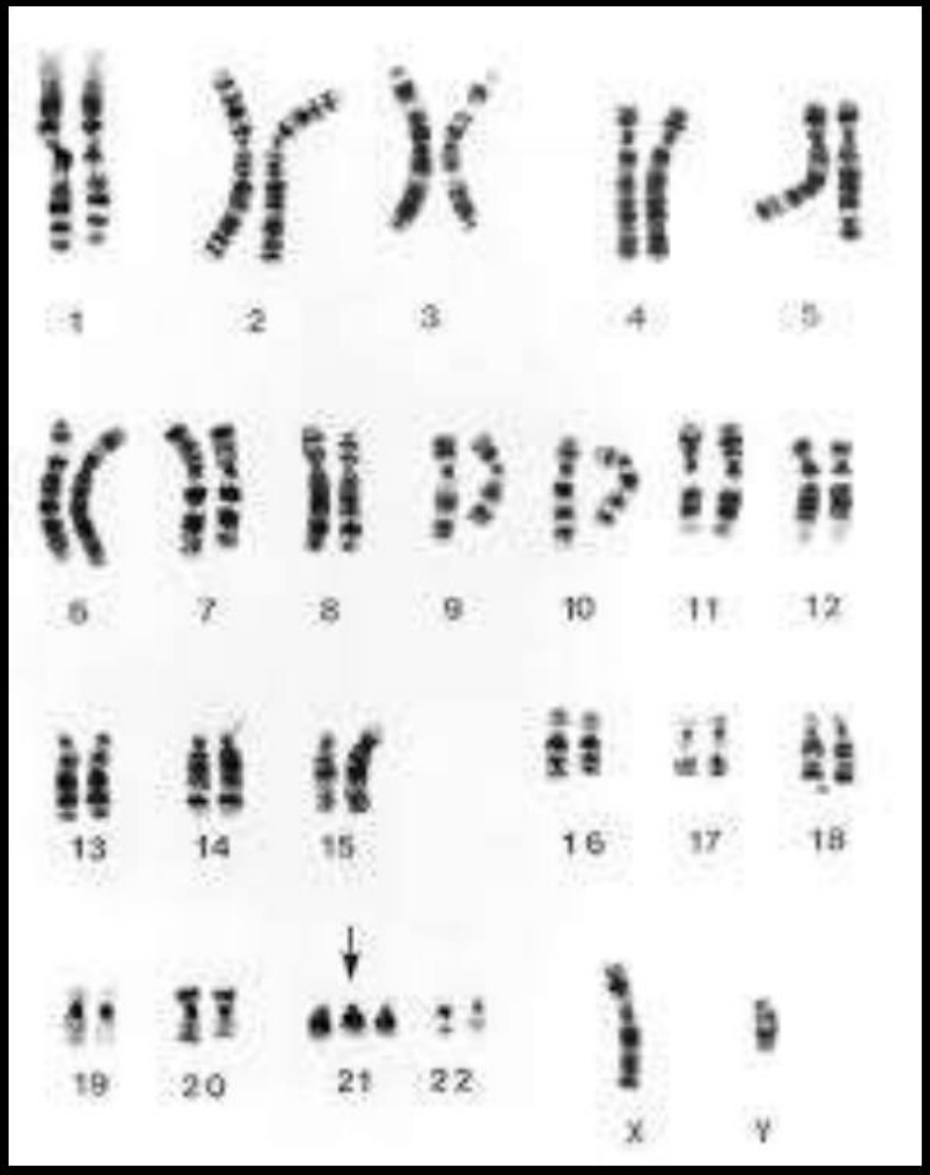
2/ Structural Abnormalities

Deletions: A portion of the chromosome is missing or deleted.

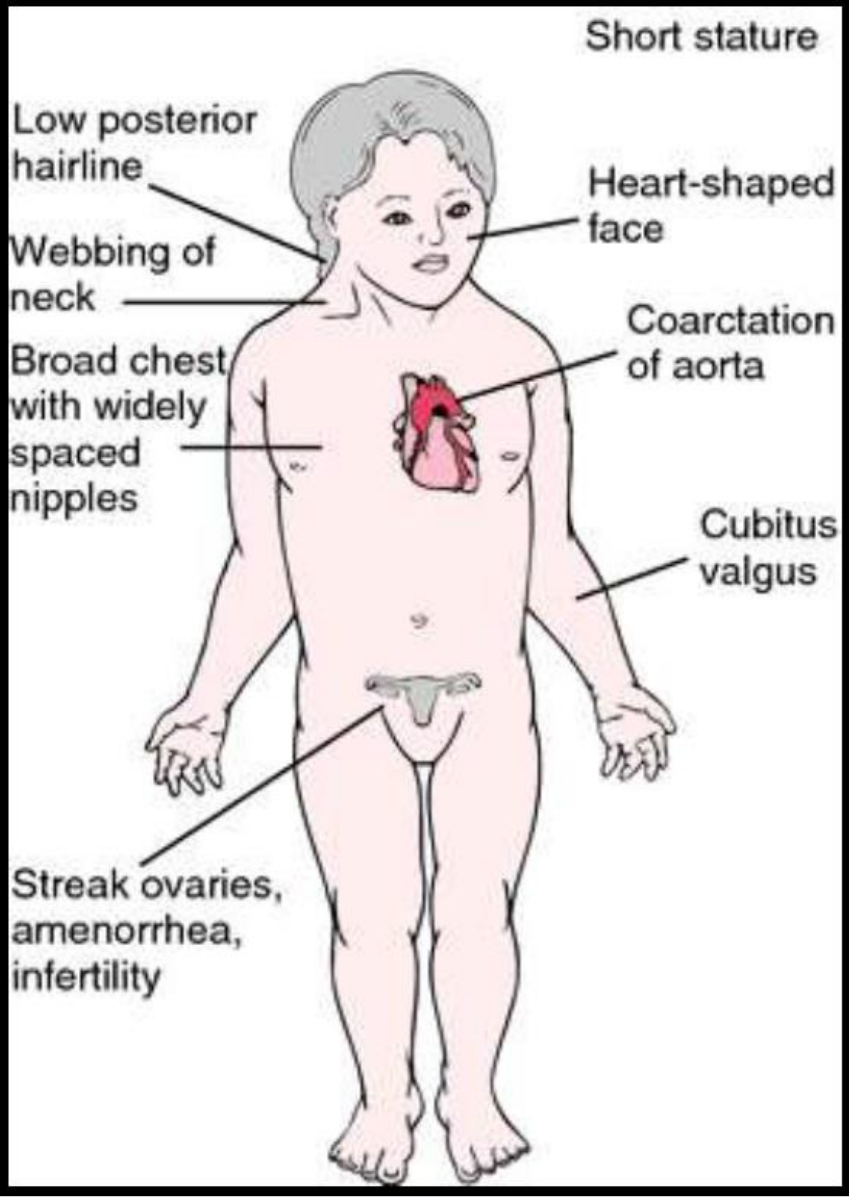
Duplications or segmental duplications: A portion of the chromosome is duplicated, resulting in extra genetic material.

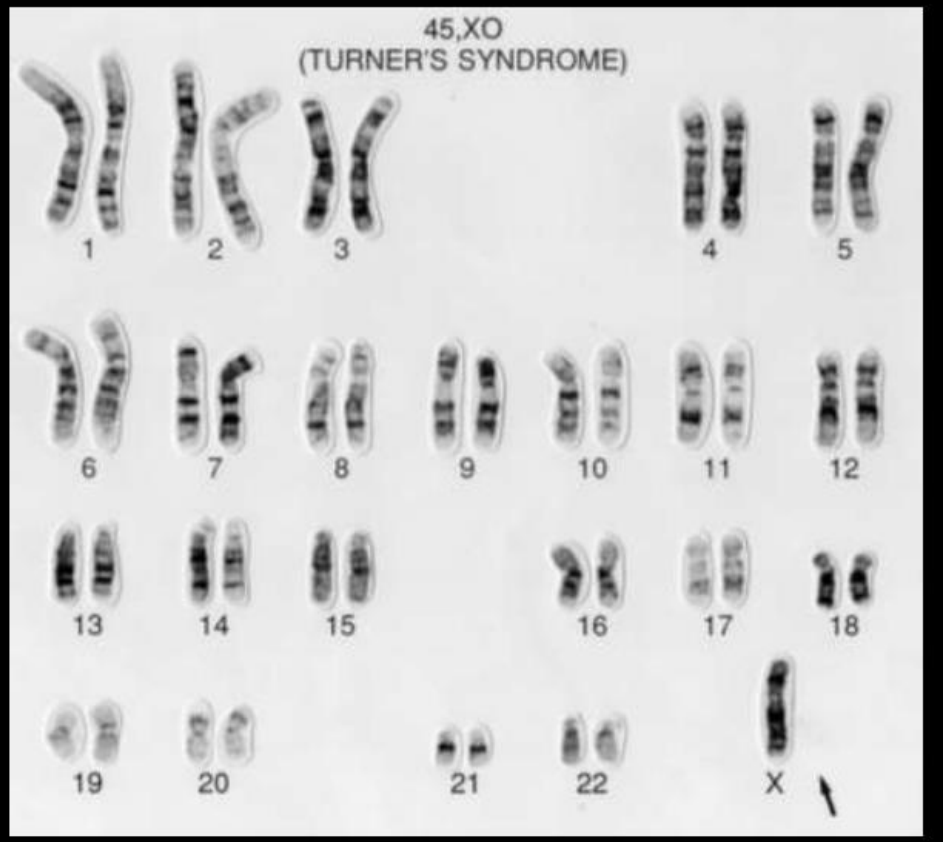
Translocations: A portion of one chromosome is transferred to another chromosome. 2 main types of translocation: a/ reciprocal translocation: segments from two different chromosomes have been exchanged; b/ Robertsonian translocation: an entire chromosome has been attached to another at the centromere.

⇒ Down Syndrome



⇒ Turner Syndrome





Cri-du-chat-Syndrome

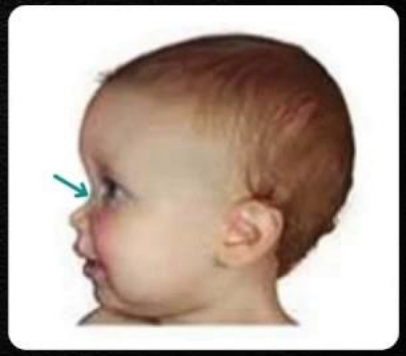
MEDVIZZ

Characteristics

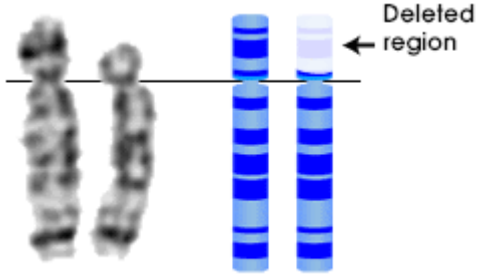
Severe developmental delay and cognitive deficits and distinctive facial abnormalities

Round face, low-set ears

Microcephaly



Hypoplastic nasal bridge



Cri-du-chat Chromosome 5 pair

AUTOSOMAL DISORDERS

Common Aneuploidies

- Trisomy 21 (Down syndrome)
- Trisomy 18 (Edward syndrome)
- Trisomy 13 (Patau syndrome)

Structural Abnormalities: Deletion Syndromes

- Cri du Chat syndrome (5p-)

Structural Abnormalities: Microdeletion Syndromes

- Di George syndrome (22q11)
- Prader-Willi syndrome (pat 15q11-q13)
- Angelman syndrome (mat 15q11-q13)

Structural Abnormalities: Trinucleotide Expansion Disorders

- Huntington Disease (4p16.3)
- Myotonic Dystrophy (19q13.2)
- Freidreich Ataxia (9q13)

SEX CHROMOSOMAL DISORDERS

Common Aneuploidies

- Klinefelter syndrome (47,XXY)
- 47,XYY syndrome
- Turner syndrome (45,X and variants)

Structural Abnormalities

- Fragile X syndrome (trinucleotide expansion; Xq27.3)
- Sex Reversal (deletion, translocation; Yp11.32)

Gene Mutations

- Change in the **nucleotide sequence** of a gene
- May only involve a single nucleotide
- May be due to **copying errors**, **chemicals**, **viruses**, etc.

Types of Gene Mutations

- Include:
  - Substitutions
  - Insertions
  - Deletions

Point Mutations

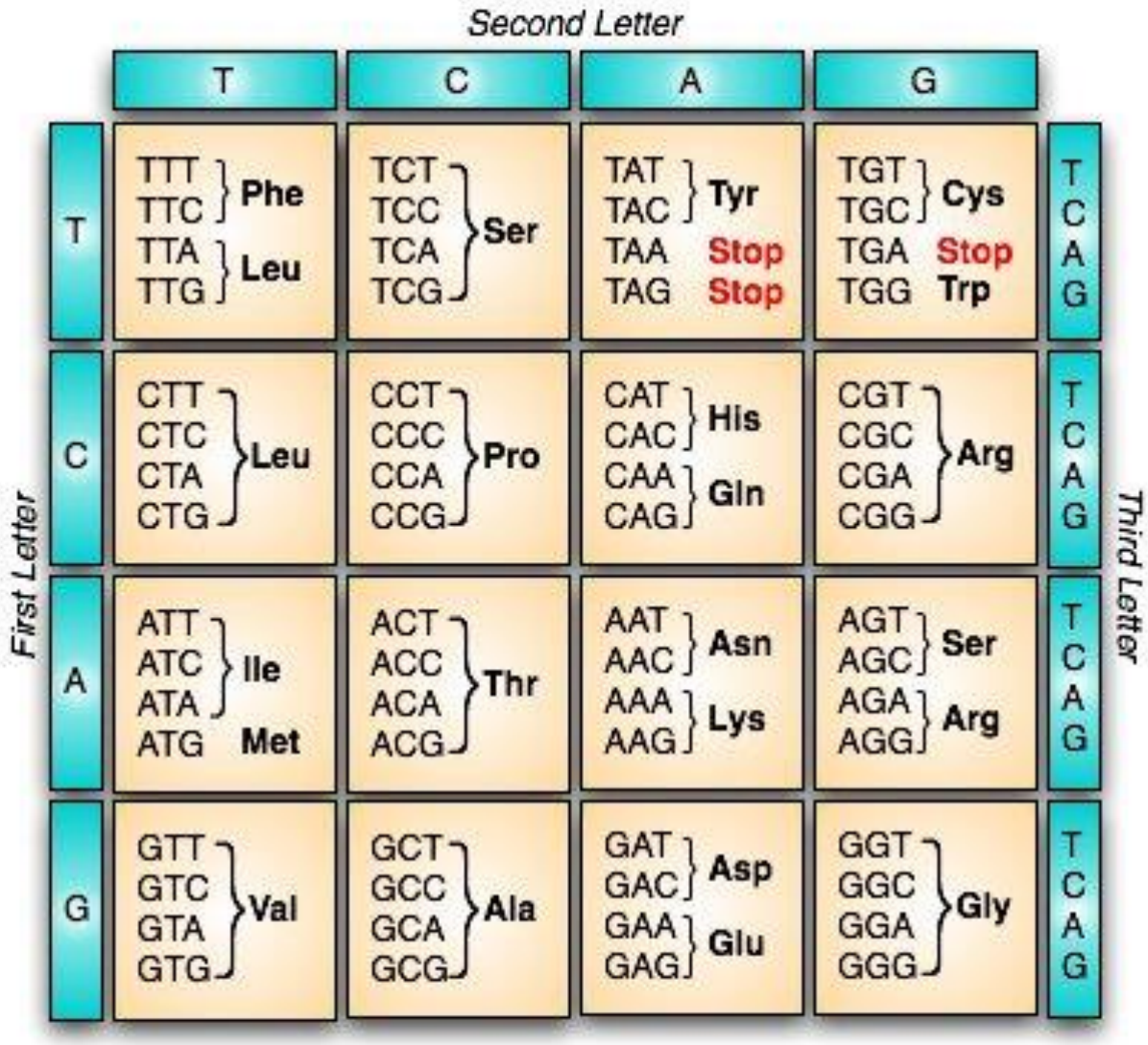
- Change of a single nucleotide
- Includes the deletion, insertion, or substitution of ONE nucleotide in a gene

Point Mutations

- Substitution of 1 base for another

- If purine (A/G) or pyrimidine (T/C) substitutes for itself = transition substitution
- If purine substitutes for pyrimidine or vice versa = transversion substitution

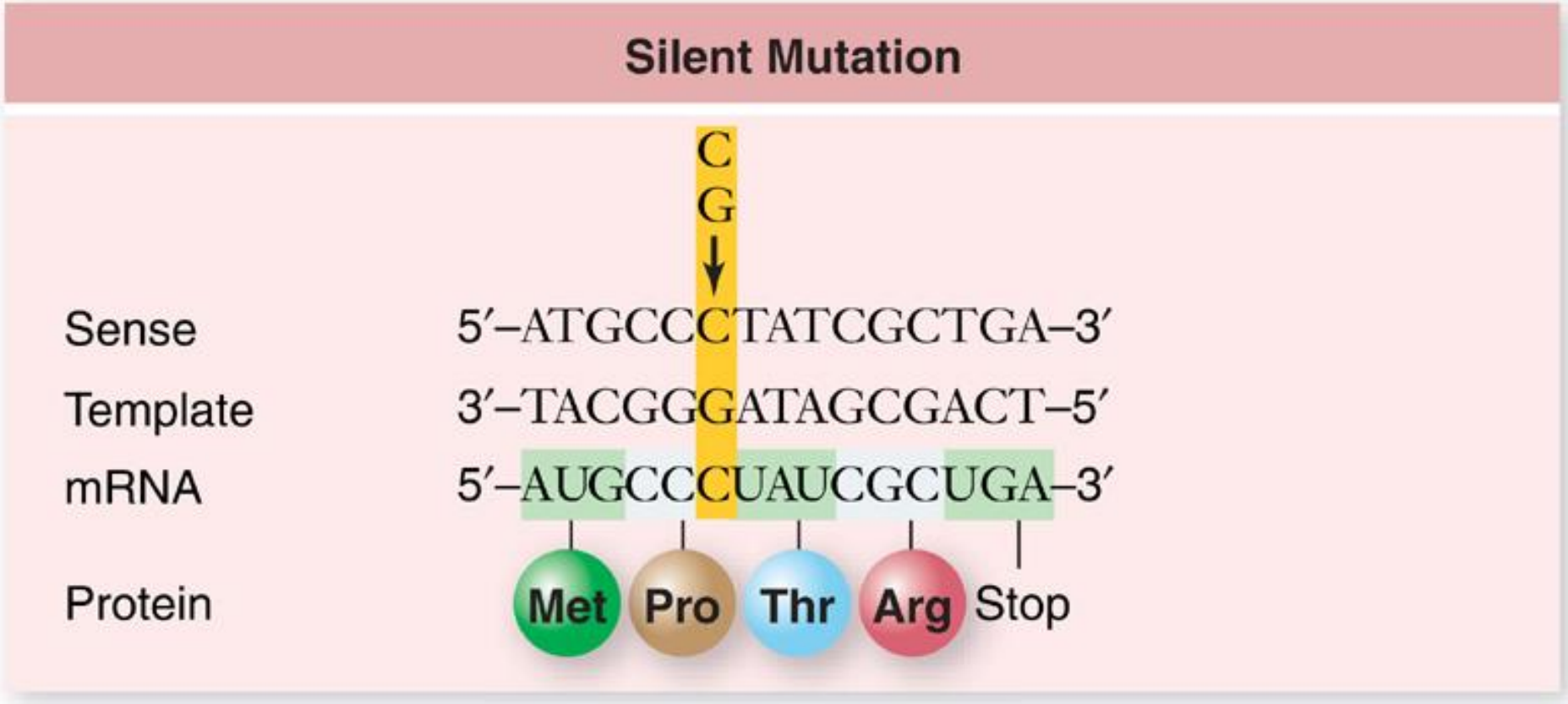
Genetic code



Results of point mutations

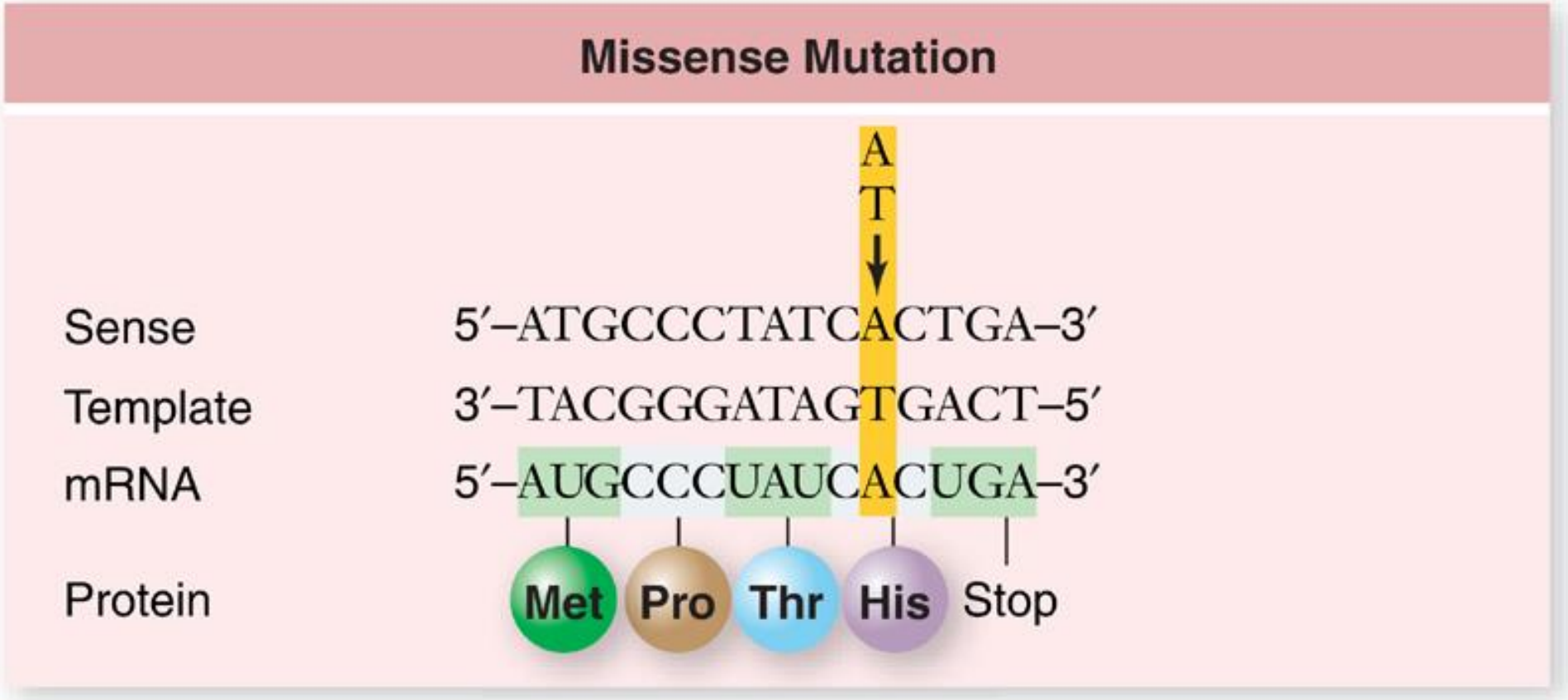
- **Silent mutations** = due to redundancy of the Genetic Code, some point mutations are silent – do not code for a different amino acid
- **Missense mutations** = produces change in amino acid in protein but does not change the function of the protein
- **Nonsense mutations** = produces a STOP codon in the midst of the mRNA transcript; can produce a non-functional protein

Silent mutation



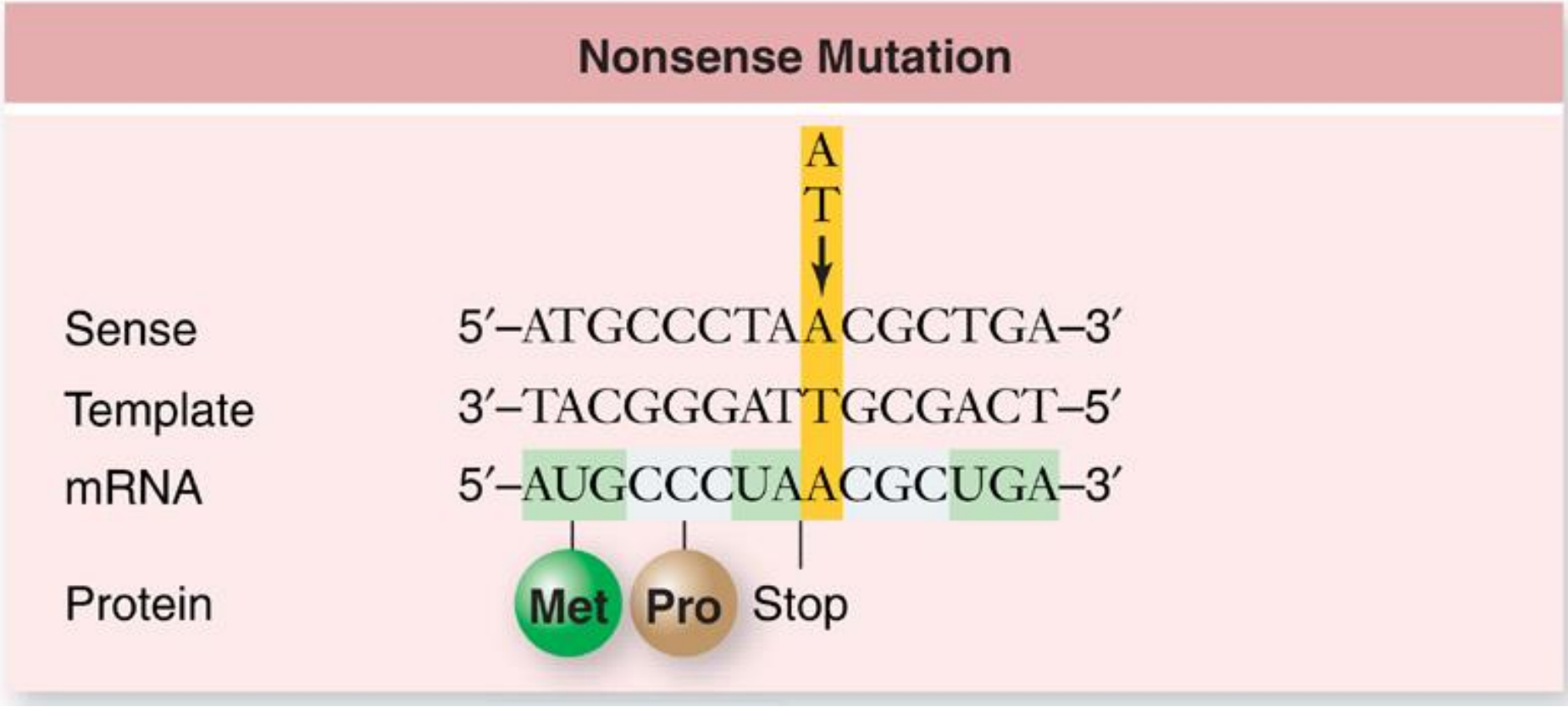
Due to redundancy of Genetic Code, no change in amino acid sequence is produced

Missense mutation



Missense mutation produces a change in amino acid sequence in protein product (Histidine in for Arginine); It may change function of protein or may not.

Nonsense mutation



Nonsense mutation produces a STOP codon within the mRNA transcript leading to a truncated protein. How short the protein product depends on where the STOP codon was produced within the mRNA transcript.

Frameshift Mutation

- Inserting or deleting one or more nucleotides
- Changes the "reading frame" like changing a sentence
- Proteins built incorrectly

Triplets de bases (ADN)

ATG GGC ATT CGT AGC TAT CCA TAA AAA TATA ...

Met Gly Ile Arg Ser Tyr Pro Stop

Base mutée (ARN)

CAU

Triplets de bases (ADN)

ATG GGC ATT CGT AGC TAT CCA TAA AAA TATA ...

Met Gly Ile Arg Ser Tyr Pro Stop

Base mutée (ARN)

GGA

Triplets de bases (ADN)

ATG GGC ATT CGT AGC TAT CCA TAA AAA TATA ...

Met Gly Ile Arg Ser Tyr Pro Stop

Base mutée (ARN)

UUA

Triplets de bases (ADN)

ATG GGC ATT CGT AGC TAT CCA TAA AAA TATA ...

Met Gly Ile Arg Ser Tyr Pro Stop

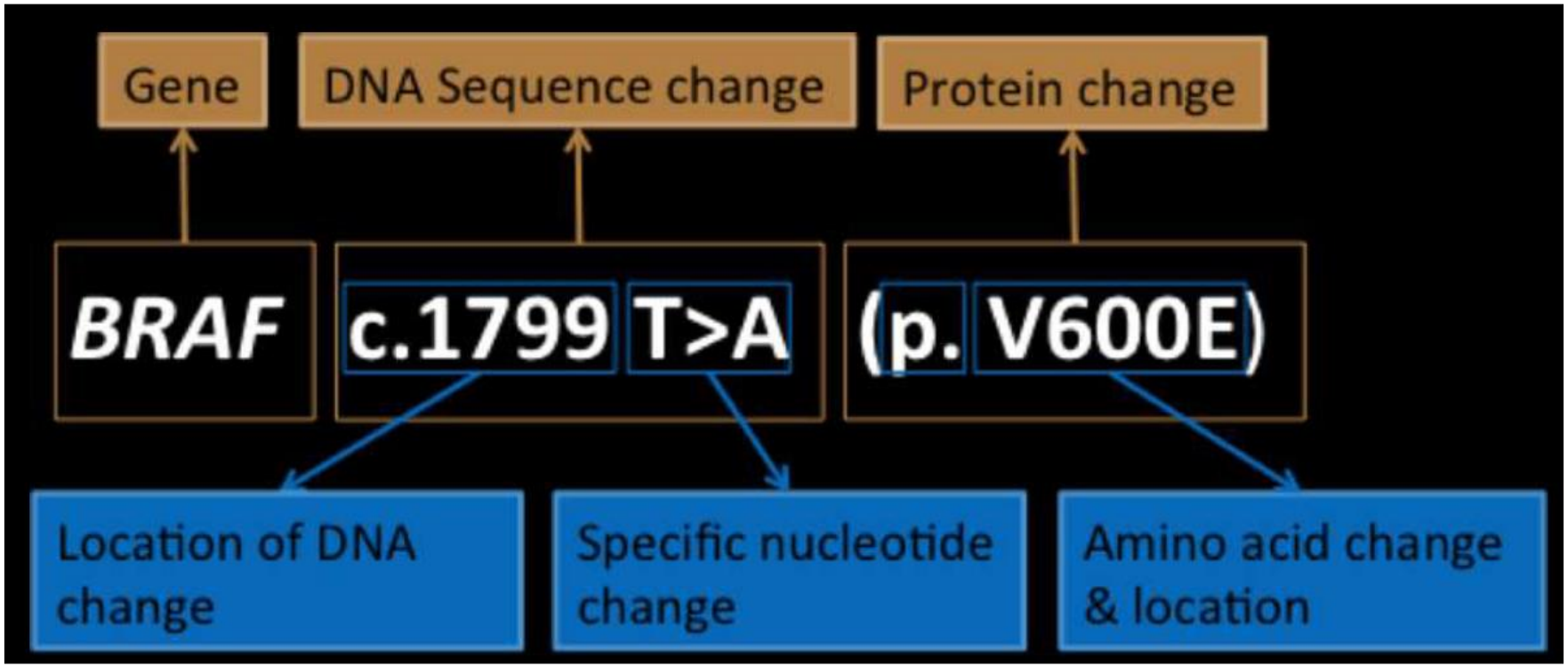
Base mutée (ARN)

UAA

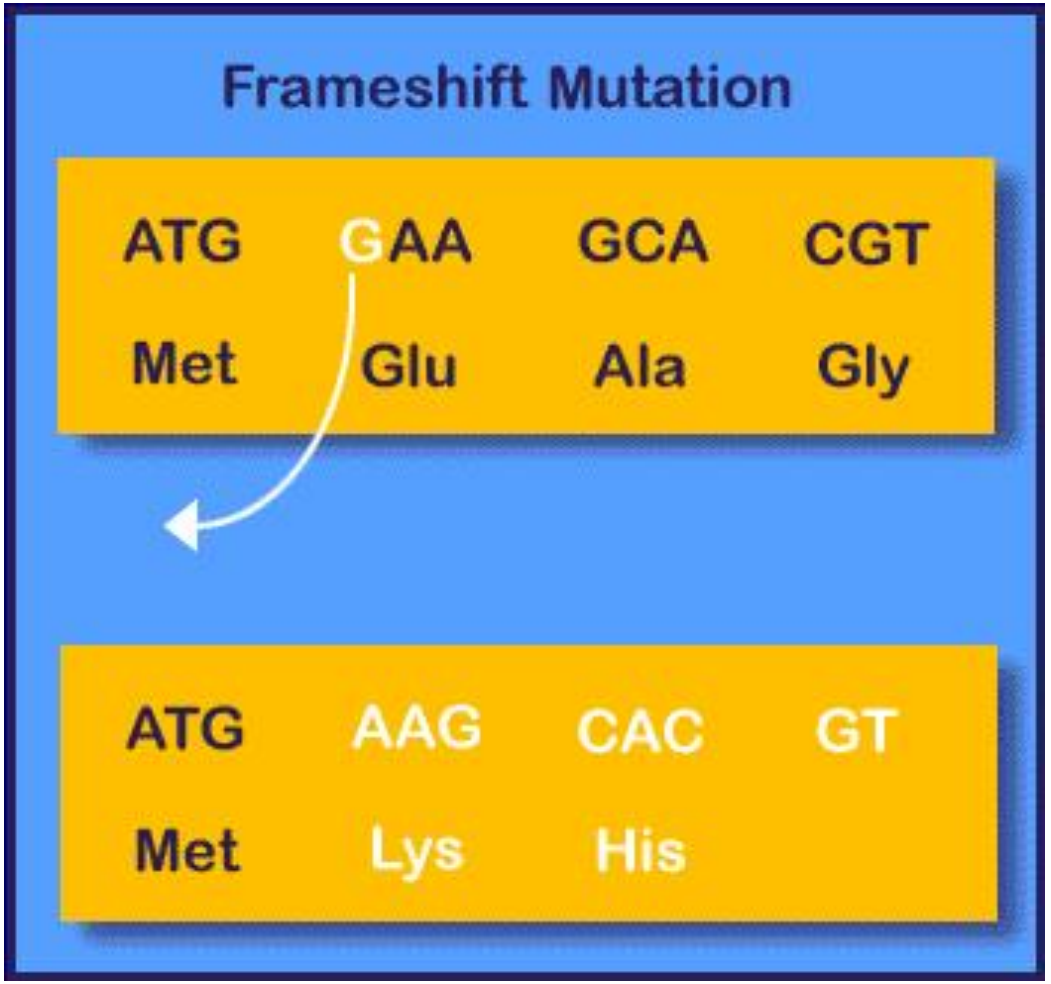
Triplets de bases (ADN)

ATG GGC ATT CGT AGC TAT CCA TAA AAA TATA ...

Met Gly Ile Arg Ser Tyr Pro Stop


Gene
DNA Sequence change
Protein change
BRAF
c.1799 T>A
(p. V600E)
Location of DNA change
Specific nucleotide change
Amino acid change & location

Amino Acid Sequence Changed

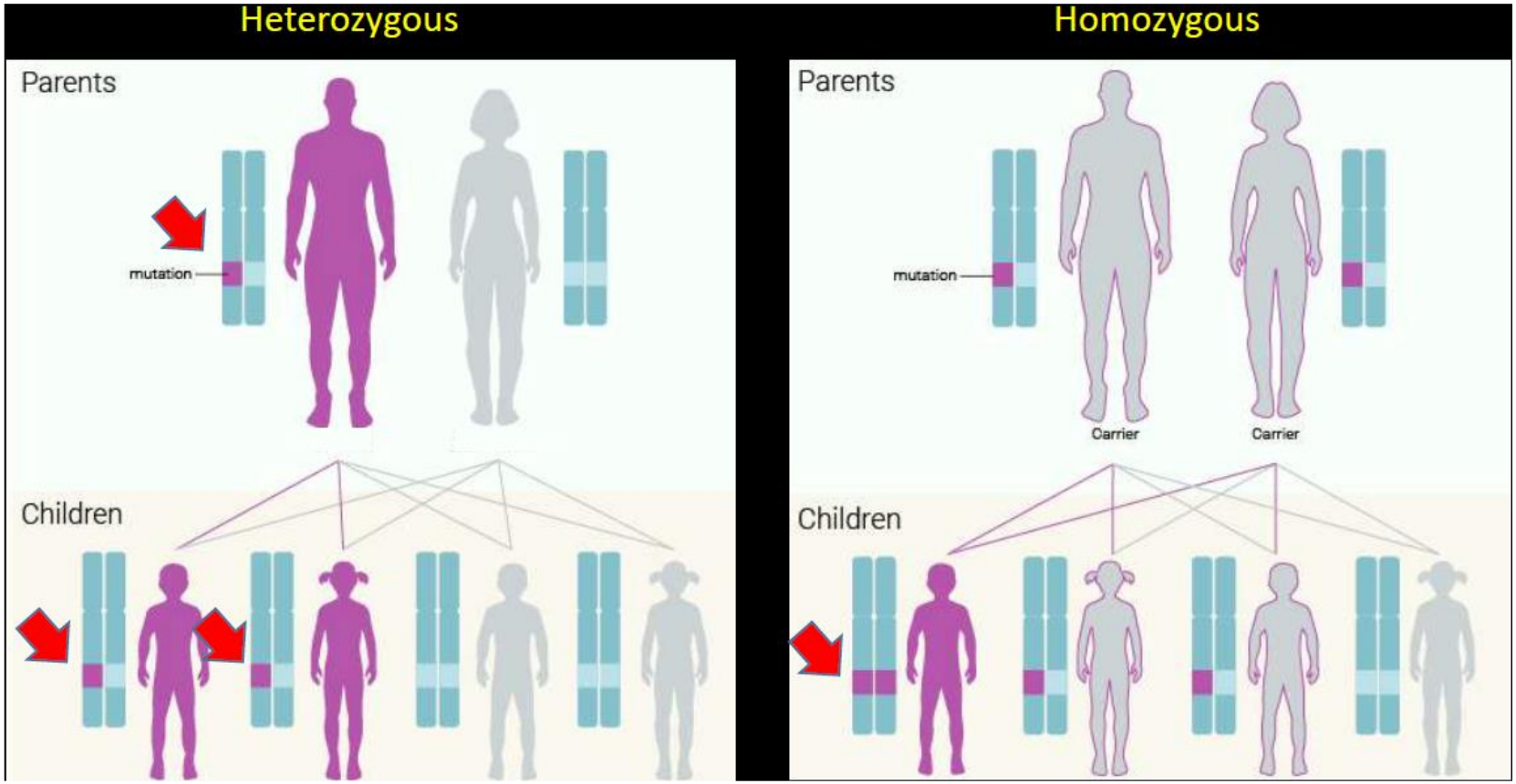


Mutations are classified by effect on protein function

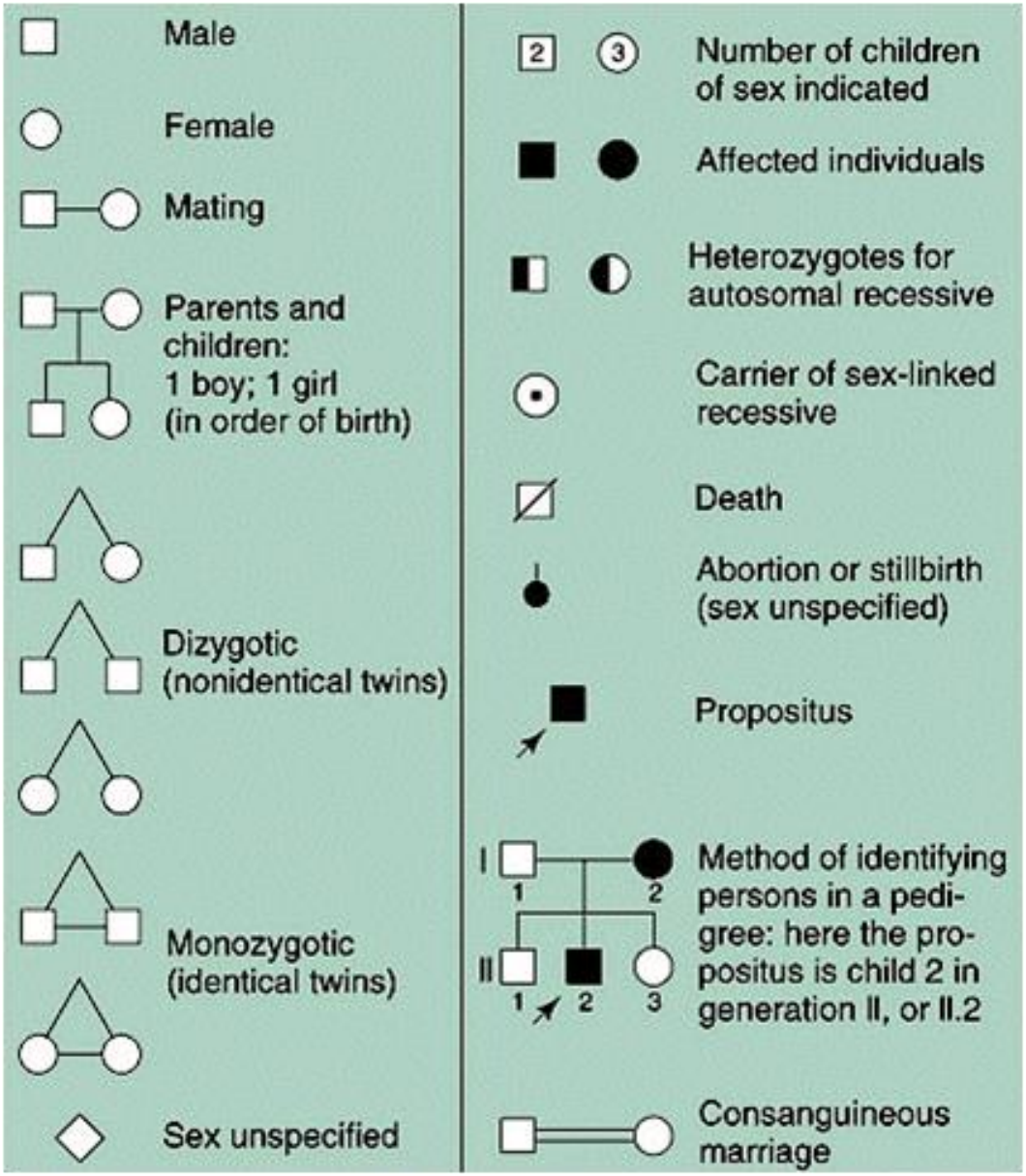
- **loss-of-function (most common)**
  decreased amount normal protein, or altered cell trafficking
- **gain-of-function**
- **novel property**
- **inappropriate expression**
  ex: Oncogenes in **cancer**

Mutations result in different alleles

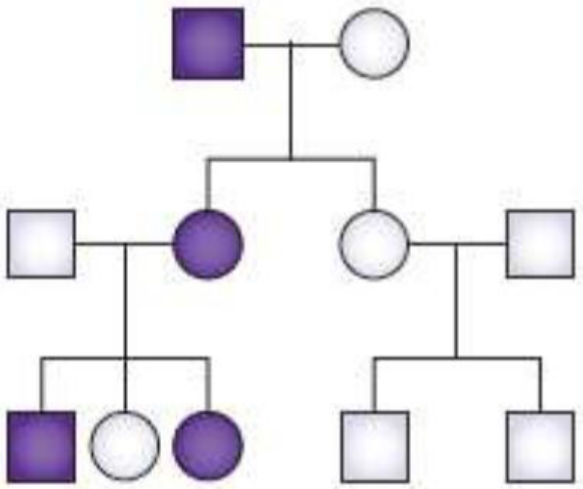
- alleles are classified as “dominant” or “recessive”
- dominant phenotypes – observable in heterozygotes
- recessive phenotypes – observable only in homozygotes


Heterozygous
Parents
mutation
Children
Homozygous
Parents
mutation
Carrier
Carrier
Children

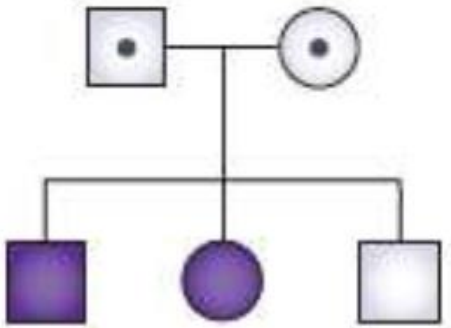
Pedigree legend in genetics



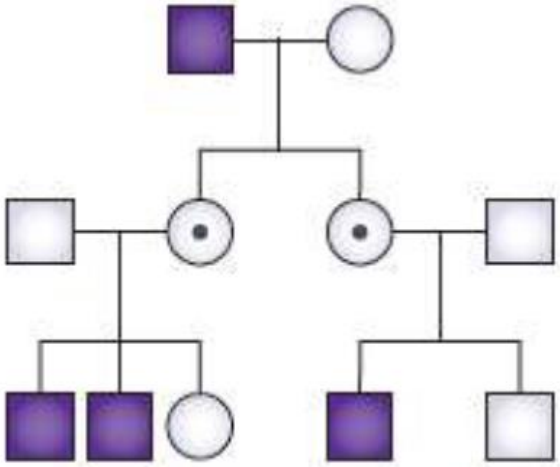
Autosomal dominant



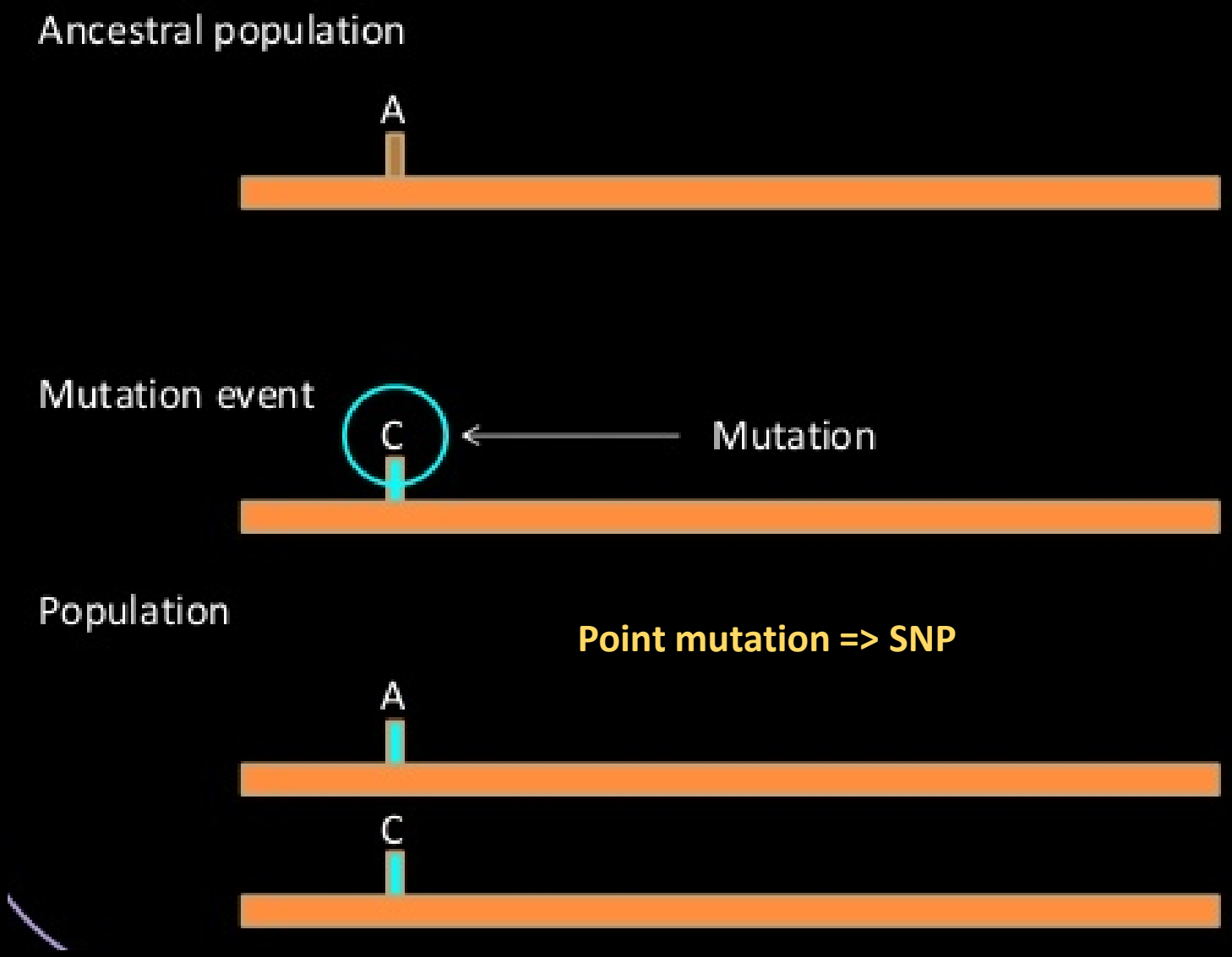
Autosomal recessive



X-linked recessive



Multifactorial genetic disorders

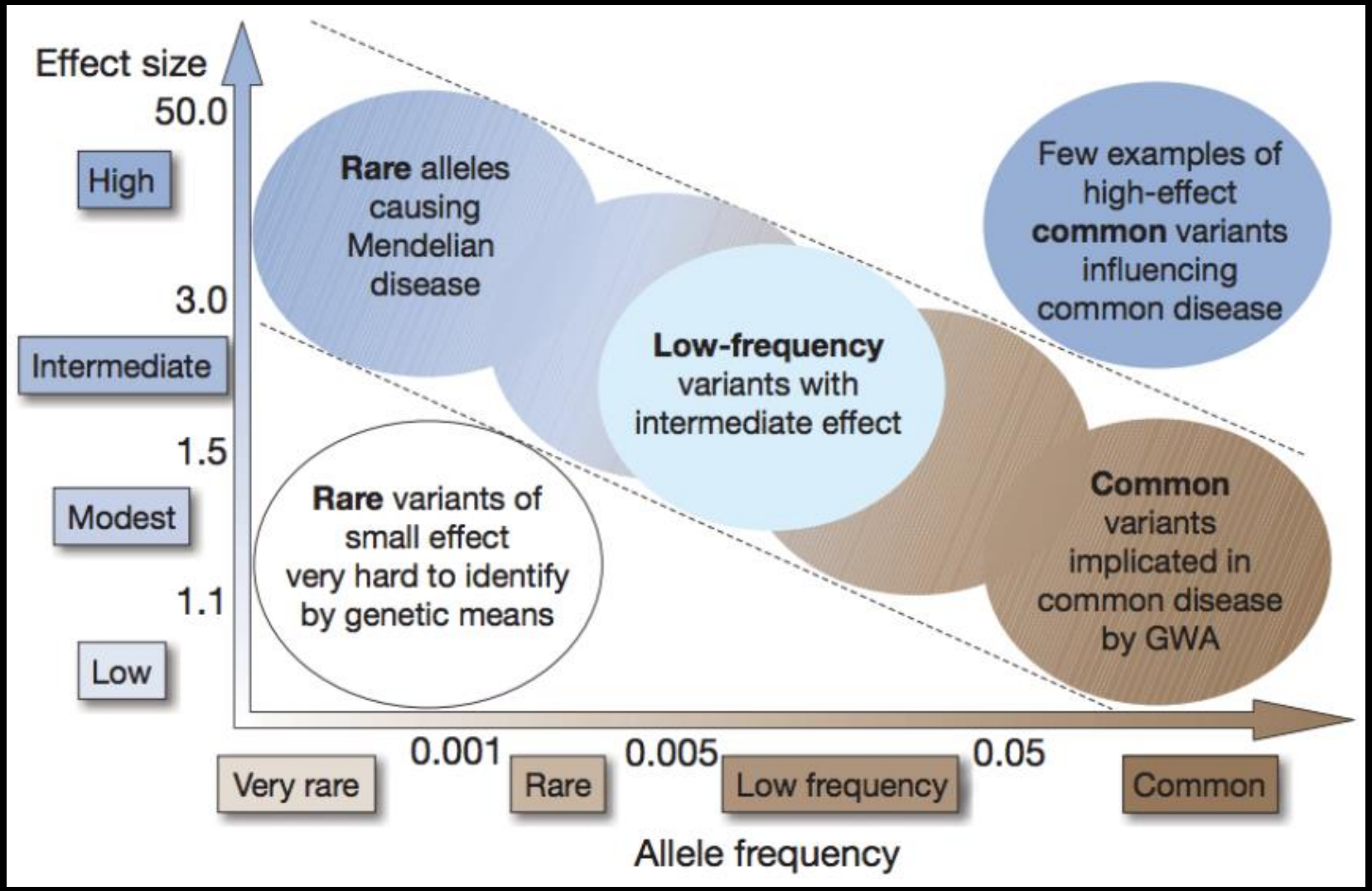


Mutations that proliferate are 'SNPs'

- Single Nucleotide Polymorphisms
- The most common type of variation in DNA
- Substitution of 1 nucleotide for another
- 2/3 SNPs involve C-> T
- Definition is evolving:
  - Old definition: SNPs must be seen in 1% of the population
  - SNPs occur ~ every 300 bp
  - Therefore ~ 10 million SNPs in the human genome

Multifactorial genetic disorders

❖ **Main assumption: A common human disease (e.g. type 2 diabetes, obesity, cancer, Alzheimer disease, Parkinson…) is due to frequent mutations (with a minor allele frequency)**

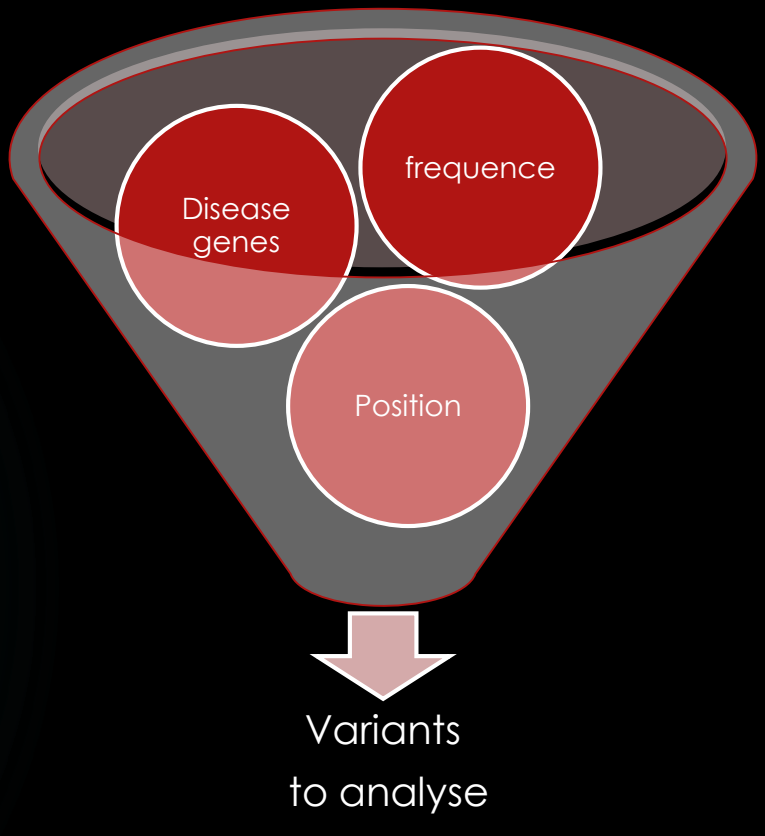


Rare genetic disorders

- Genetic disorder = when the illness is caused by one or more abnormalities in the genome
- Rare genetic disorder = when the abnormality is monogenic /located on one gene
- >5000 human diseases are caused by rare genetic disorders

- Only one abnormality can cause the illness!!

How to filter NGS data ?



Diagnosis: ACMG criteria

- ACMG = American College of Medical Genetics and Genomics
- Consensus: criteria to classify pathogenic variants

Interest variants

ACMG criteria

Pathogenic P
Likely Pathogenic LP

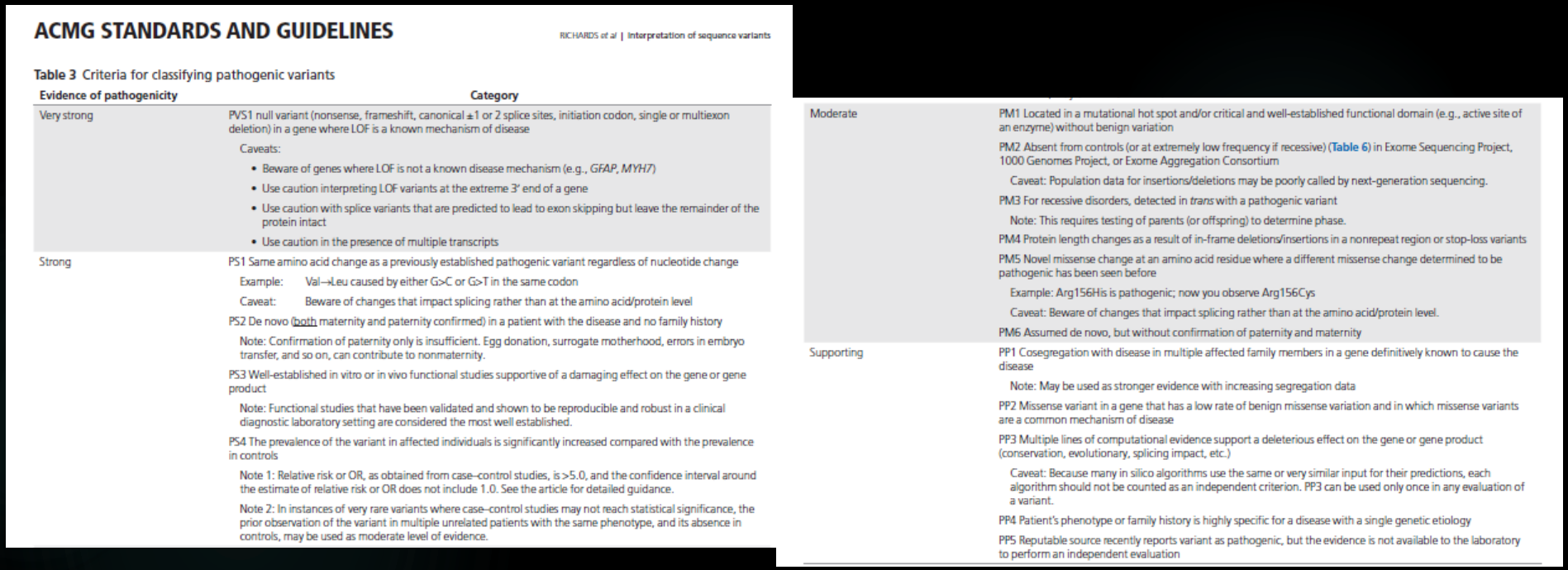
OR

Variant of Uncertain Significance VUS

Causative variant

No diagnosis

Diagnosis: ACMG criteria



ACMG STANDARDS AND GUIDELINES

Table 3 Criteria for classifying pathogenic variants

Evidence of pathogenicity	Category
Very strong	PVS1 null variant (nonsense, frameshift, canonical ±1 or 2 splice sites, initiation codon, single or multiexon deletion) in a gene where LOF is a known mechanism of disease Caveats: • Beware of genes where LOF is not a known disease mechanism (e.g., *GFAP*, *MYH7*) • Use caution interpreting LOF variants at the extreme 3′ end of a gene • Use caution with splice variants that are predicted to lead to exon skipping but leave the remainder of the protein intact • Use caution in the presence of multiple transcripts
Strong	PS1 Same amino acid change as a previously established pathogenic variant regardless of nucleotide change Example: Val→Leu caused by either G>C or G>T in the same codon Caveat: Beware of changes that impact splicing rather than at the amino acid/protein level PS2 De novo (both maternity and paternity confirmed) in a patient with the disease and no family history Note: Confirmation of paternity only is insufficient. Egg donation, surrogate motherhood, errors in embryo transfer, and so on, can contribute to nonmaternity. PS3 Well-established in vitro or in vivo functional studies supportive of a damaging effect on the gene or gene product Note: Functional studies that have been validated and shown to be reproducible and robust in a clinical diagnostic laboratory setting are considered the most well established. PS4 The prevalence of the variant in affected individuals is significantly increased compared with the prevalence in controls Note 1: Relative risk or OR, as obtained from case–control studies, is >5.0, and the confidence interval around the estimate of relative risk or OR does not include 1.0. See the article for detailed guidance. Note 2: In instances of very rare variants where case–control studies may not reach statistical significance, the prior observation of the variant in multiple unrelated patients with the same phenotype, and its absence in controls, may be used as moderate level of evidence.
Moderate	PM1 Located in a mutational hot spot and/or critical and well-established functional domain (e.g., active site of an enzyme) without benign variation PM2 Absent from controls (or at extremely low frequency if recessive) (Table 6) in Exome Sequencing Project, 1000 Genomes Project, or Exome Aggregation Consortium Caveat: Population data for insertions/deletions may be poorly called by next-generation sequencing. PM3 For recessive disorders, detected in *trans* with a pathogenic variant Note: This requires testing of parents (or offspring) to determine phase. PM4 Protein length changes as a result of in-frame deletions/insertions in a nonrepeat region or stop-loss variants PM5 Novel missense change at an amino acid residue where a different missense change determined to be pathogenic has been seen before Example: Arg156His is pathogenic; now you observe Arg156Cys Caveat: Beware of changes that impact splicing rather than at the amino acid/protein level. PM6 Assumed de novo, but without confirmation of paternity and maternity
Supporting	PP1 Cosegregation with disease in multiple affected family members in a gene definitively known to cause the disease Note: May be used as stronger evidence with increasing segregation data PP2 Missense variant in a gene that has a low rate of benign missense variation and in which missense variants are a common mechanism of disease PP3 Multiple lines of computational evidence support a deleterious effect on the gene or gene product (conservation, evolutionary, splicing impact, etc.) Caveat: Because many in silico algorithms use the same or very similar input for their predictions, each algorithm should not be counted as an independent criterion. PP3 can be used only once in any evaluation of a variant. PP4 Patient's phenotype or family history is highly specific for a disease with a single genetic etiology PP5 Reputable source recently reports variant as pathogenic, but the evidence is not available to the laboratory to perform an independent evaluation

Standards and guidelines for the interpretation of sequence variants: a joint consensus recommendation of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology; Genet Med. 2015 May;17(5)

Where find data?

- Databases: HGMD, clinVar, GnomAD….
- Data about known variants, for example:
  - Presence of another variant at the same locus in a gene
  - Allelic frequence in general populations
  - Evidence of deleterious effect on the gene or on the gene product
  - Scientific publication about mutations
  - *In vivo* functional studies

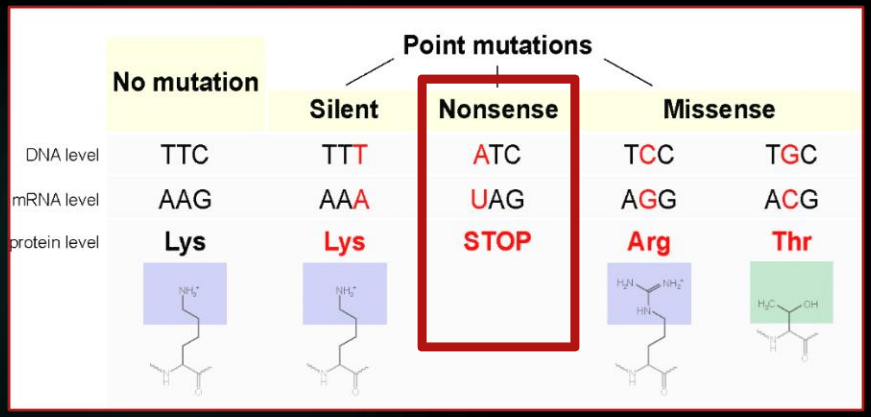
- Alamut software is a convenient access to several databases of known variants
- Warning: always check that you explore the same transcript : NM_.....

- Prediction algorithmes:
- Splice site prediction
- Nucleotide conservation prediction

All these data are needed in order to classify the variants

PVS1 criterion (Pathogenicity Very Strong)

- null variant = nonsense, frameshift, canonical ±1 or 2 splice sites, initiation codon, single or multiexon deletion



Nonsense mutation: check the Amino Acid change: *=stop

Examples:

NM_000207.2 c.184C>T, p.(Gln62*)

NM_000207.2 c.324C>G, p.(Tyr108*)

cDNA

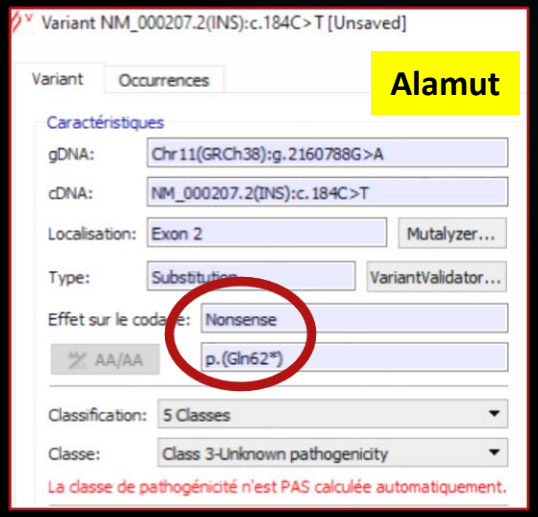
protein

PVS1 criterion

- null variant = nonsense, frameshift, canonical ±1 or 2 splice sites, initiation codon, single or multiexon deletion

Nonsense mutation: check the Amino Acid change:
*=stop
Exemples:
gene INS (AD)
NM_000207.2 c.184C>T, p.(Gln62*) ht, AD
NM_000207.2 c.324C>G, p.(Tyr108*) ht, AD

cDNA protein

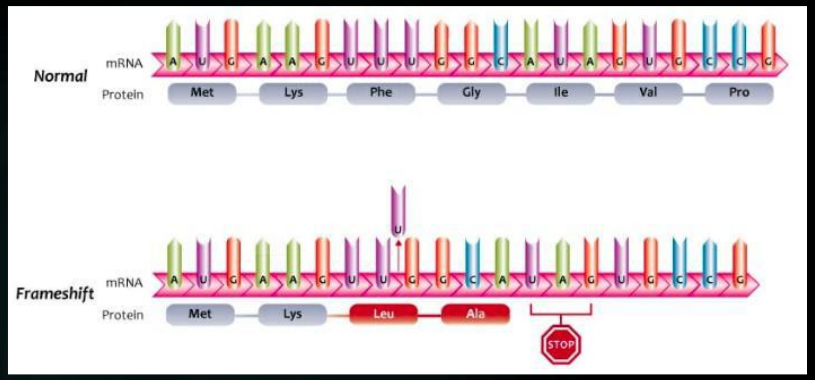


Where to check:

- NGS Annotation File
- Alamut software
- Public databases

PVS1 criterion

- null variant = nonsense, frameshift, canonical ±1 or 2 splice sites, initiation codon, single or multiexon deletion



Frameshift mutation: deletion/insertion in which the number of deleted base pairs is not divisible by three:
check the Amino Acid change and consequences
Example:
c.2711-2714del // p.(His905Alafs*34)

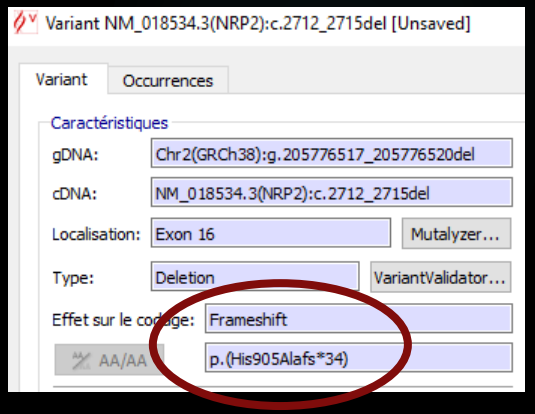
Warning: Indel of multiple of 3 nucleotides = indel of amino acid without frameshift

PVS1 criterion

- null variant = nonsense, frameshift, canonical ±1 or 2 splice sites, initiation codon, single or multiexon deletion

Alamut

Frameshift mutation: in case of insertion or deletion: check the Amino Acid change and consequences
Exemple:
NM_018534 c.2712-2715del // p.(His905Alafs*34)



Warning: Insertion /deletion of multiple of 3 nucleotides = indel of amino acid without frameshift

Where to check:

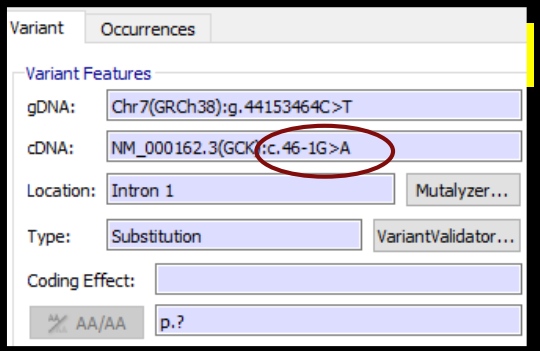
- NGS Annotation File
- Alamut software
- Public databases

PVS1 criterion

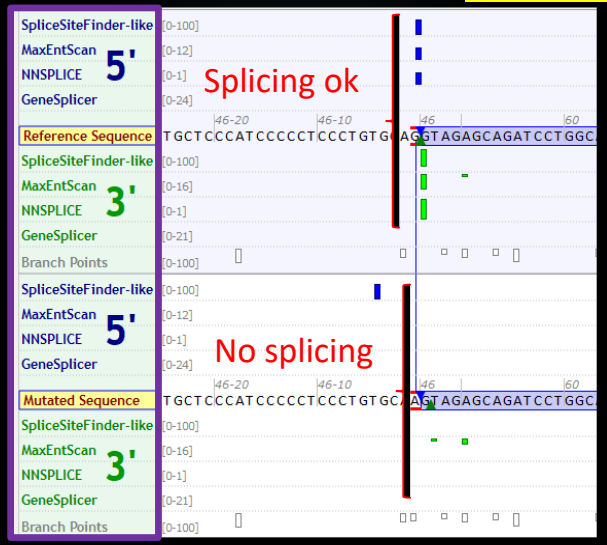
- null variant = nonsense, frameshift, canonical ±1 or 2 splice sites, initiation codon, single or multiexon deletion

Splice site variant: at the boundary of an exon and an intron .
For example: gene GCK (AD)
Intronic:
NM_000162.3 c.46-4G>A, ht

Exonic:
NM_000162.3 c.211G>C , ht
(intron start after c.211)



Alamut



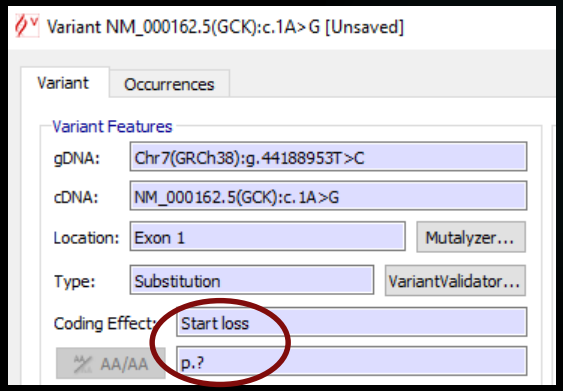
Where to check:

- Alamut software
- Splicing prediction tools

PVS1 criterion

- null variant = nonsense, frameshift, canonical ±1 or 2 splice sites, initiation codon, single or multiexon deletion

Initiation codon: first ATG = Methionine = M
exemple: c.1A>G, p.?



Alamut

single or multi-exon deletion

PS1 criterion (Pathogenicity Strong)

Same amino acid change as a previously established pathogenic variant regardless of nucleotide change

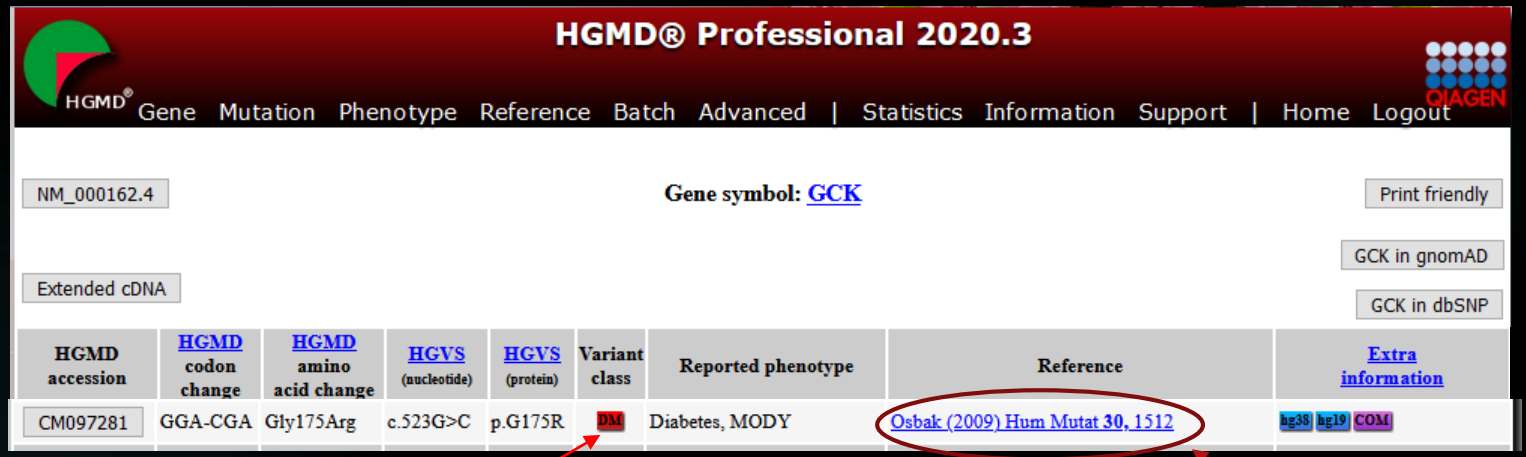
example: Val -> Leu caused by either G > C or G > T in the same codon

Val	Leu	
GTG	CTG	Pathogenic variant present in databases
	TTG	Interest Variant

Can been checked in Alamut (database ClinVar) and/or in HGMD

PS1 criterion (Pathogenicity Strong)

HGMD



DM =Disease Mutation
DM= PS1! (read publication)

Publication

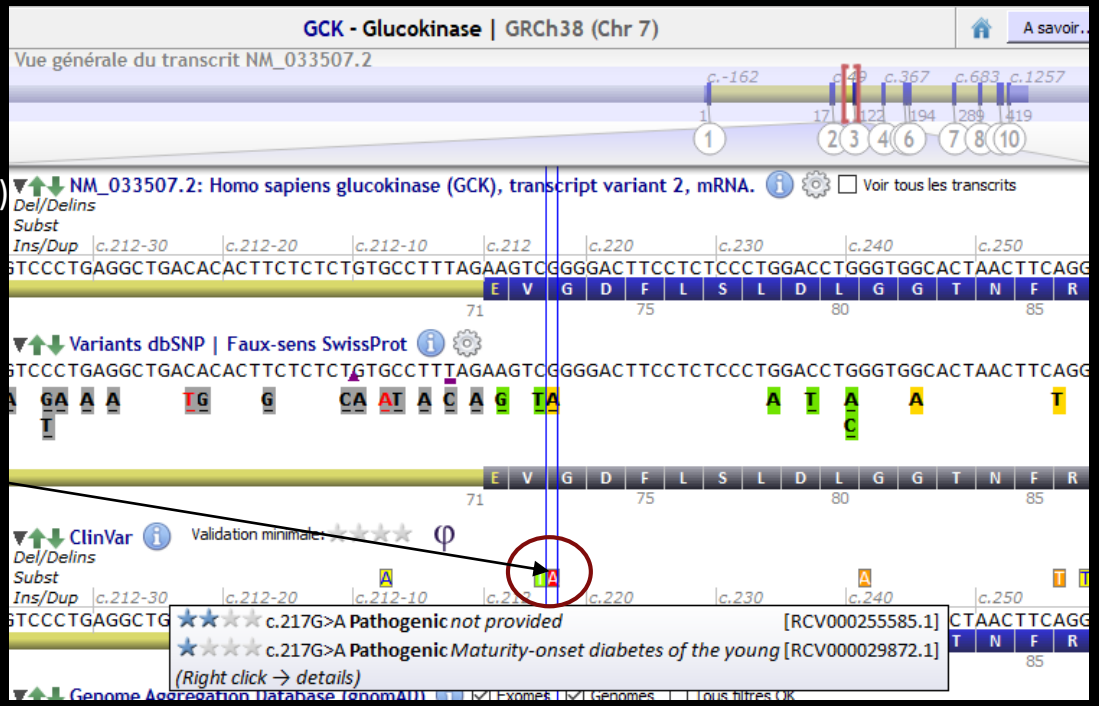
Variant: NM_000162.5(GCK):c.523G>C // p.Gly175Arg
Already present in HGMD (in this case with the same nucleotide change)

In HGMD, check by categories: Missense/nonsense, splicing mutations, insertions, deletions

PS1 criterion (Pathogenicity Strong)

ClinVar
via Alamut

Variant:
Gene GCK (AD/AR)
NM_000162.5(GCK):c.217G>A, p.(Gly73Arg)



Red =pathogenic
Orange = likely pathogenic
Green, uncertain signifiance
Red = PS1! (read publication)

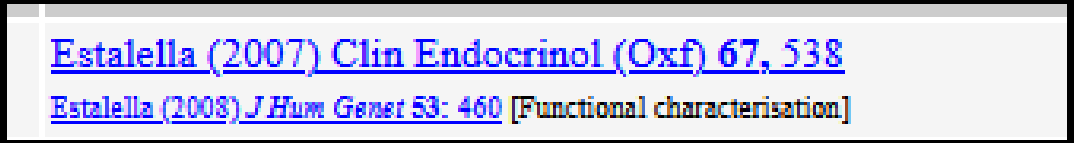
PS3 criterion (Pathogenicity Strong)

Well-established in vitro or in vivo functional studies supportive of a damaging effect on the gene or gene product

HGMD

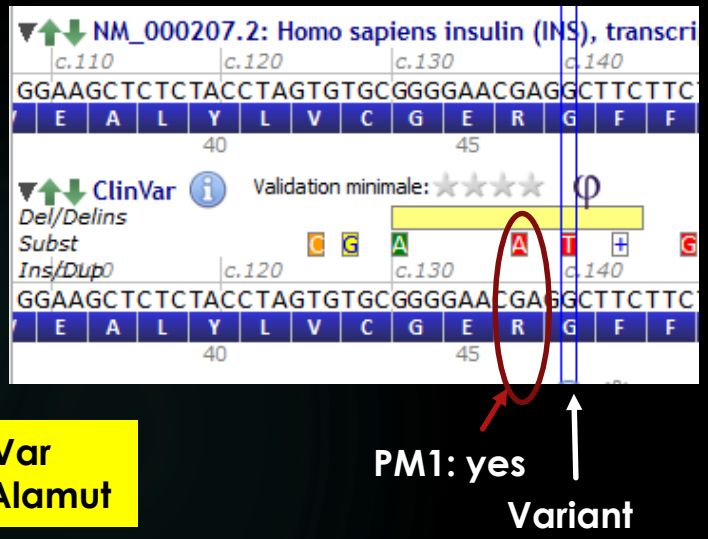
DM =Disease Mutation

reference with [Functional characterisation]= PS3! (read publication)



PM1 criterion (Pathogenicity Moderate)

PM1 Located in a mutational hot spot and/or critical and well-established functional domain (e.g., active site of an enzyme) without benign variation



ClinVar via Alamut

Hotspot: pathogenic variant (red) in one residue before or after the interest variant without benign variant (green)

PM1 criterion (Pathogenicity Moderate)

PM1 Located in a mutational hot spot and/or critical and well-established functional domain (e.g., active site of an enzyme) without benign variation

HGMD

HGMD accession	HGMD codon change	HGMD amino acid change	HGVS (nucleotide)	HGVS (protein)	Variant class	Reported phenotype	Reference	Extra information
CM1812672	TTA-TCA	Leu662Ser	c.1985T>C	p.L662S	DM	Obesity	Kleinendorst (2018) J Med Genet 55, 578 Kleinendorst (2017) *BMJ Case Rep* 2017: [Additional report]	hg38 hg19 dbSNP
CM070187	CAT-CCT	His684Pro	c.2051A>C	p.H684P	DM	Obesity, early-onset	Farooqi (2007) N Engl J Med 356, 237 Kimber (2008) *Endocrinology* 149: 6043 [Functional characterisation] Clément (2018) *Nat Med* 24: 551 [Additional case report] 2 more reference(s)...	hg38 hg19 dbSNP gnomAD
CM168926	TCT-TTT	Ser723Phe	c.2168C>T	p.S723F	DM	Obesity, severe	Hannema (2016) Horm Res Paediatr 85, 412 Kleinendorst (2017) *BMJ Case Rep* 2017: [Additional report] Kleinendorst (2018) *J Med Genet* 55: 578 [Additional report]	hg38 hg19

Example:
NM_002303.5(LEPR):c.2047C>T, p.(His683Tyr), ht

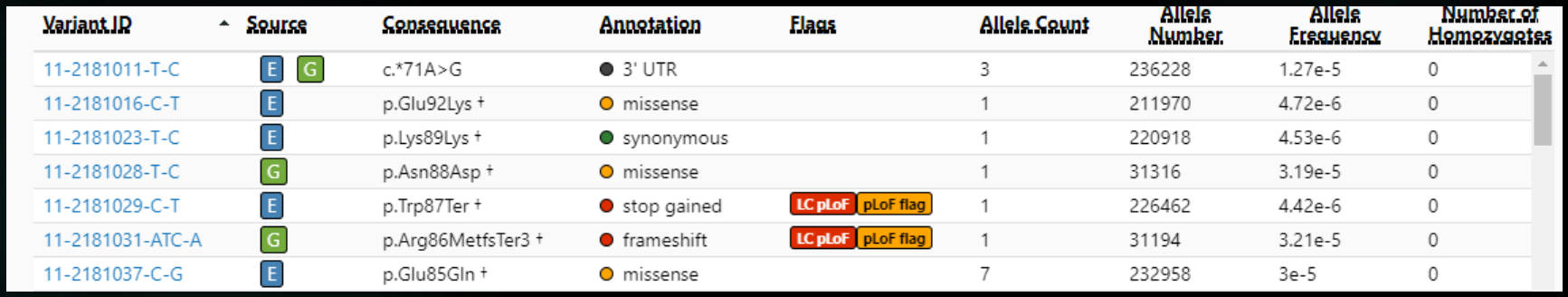
In case of misense or insertion or deletion, check missense variants

Hotspot: pathogenic variant  **e residue before or after the interest variant without benign variant (green)**

PM2 criterion (Pathogenicity Moderate)

PM2 Absent from controls (or at extremely low frequency if recessive) in GnomAD

Where to check? **gnomAD browser**



Variant ID	Source	Consequence	Annotation	Flags	Allele Count	Allele Number	Allele Frequency	Number of Homozygotes
11-2181011-T-C	E G	c.*71A>G	3' UTR		3	236228	1.27e-5	0
11-2181016-C-T	E	p.Glu92Lys †	missense		1	211970	4.72e-6	0
11-2181023-T-C	E	p.Lys89Lys †	synonymous		1	220918	4.53e-6	0
11-2181028-T-C	G	p.Asn88Asp †	missense		1	31316	3.19e-5	0
11-2181029-C-T	E	p.Trp87Ter †	stop gained	LC pLoF pLoF flag	1	226462	4.42e-6	0
11-2181031-ATC-A	G	p.Arg86MetfsTer3 †	frameshift	LC pLoF pLoF flag	1	31194	3.21e-5	0
11-2181037-C-G	E	p.Glu85Gln †	missense		7	232958	3e-5	0

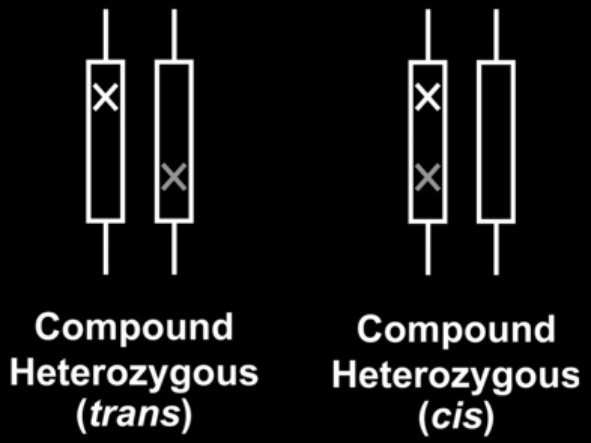
Example: NM_000207.2(INS): p.(Ile91Val), ht: PM2=yes
NM_000207.2(INS): p.(Glu92Lys), ht: PM2=no, homoz:PM2= yes

PM3 criterion (Pathogenicity Moderate)

- PM3 For recessive disorders, detected in *trans* with a pathogenic variant
- Note: This requires testing of parents (or offspring) to determine phase.

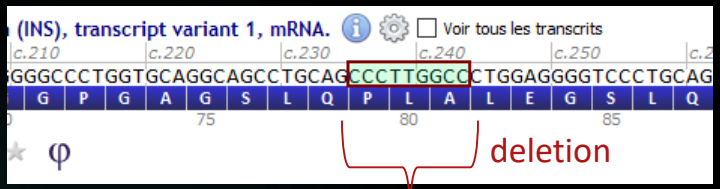
Two pathogenic variants in the same gene:

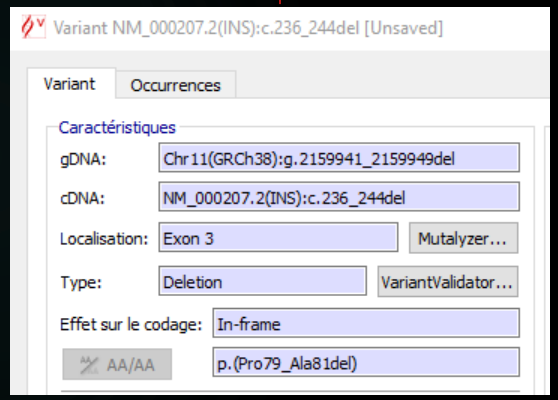
Sequencing of the parents:
- one parent is carrier of the two variants: PM3 = no
- each parent is carrier of one variant: PM3 = yes



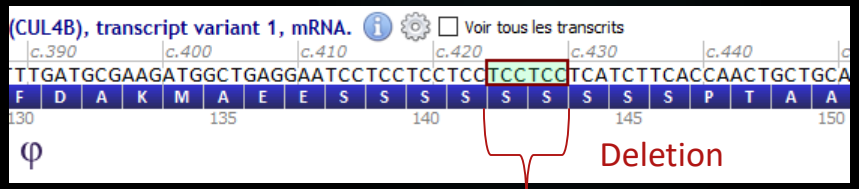
PM4 criterion (Pathogenicity Moderate)

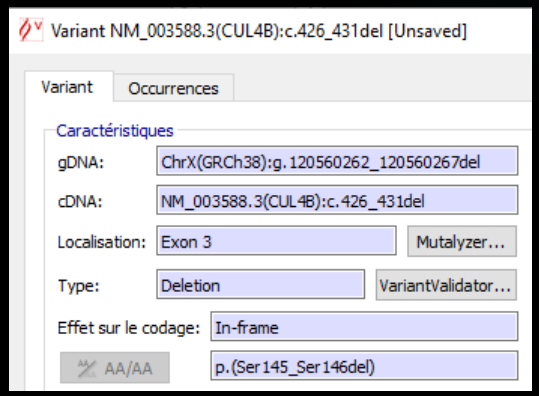
- PM4 Protein length changes as a result of in-frame deletions/insertions in a nonrepeat region or stop-loss variants





PM4: yes





Alamut

PM4: no

PM5 criterion (Pathogenicity Moderate)

- Novel missense change at an amino acid residue where a different missense change determined to be pathogenic has been seen before
- Example: Arg156His is pathogenic; now you observe Arg156Cys

- NM_000162.5(GCK):c.67T>C, p.(Phe23Leu)

HGMD

CM191975	CTG-CGG	Leu20Arg	c.59T>G	p.L20R	DM	Diabetes, gestational	Zubkova (2019) Acta Diabetol ,
CM074228	CTG-CCG	Leu20Pro	c.59T>C	p.L20P	DM	Diabetes, MODY	Estalella (2007) Clin Endocrinol (Oxf) **67**, 538
CM096803	TTC-GTC	Phe23Val	c.67T>G	p.F23V	DM	Diabetes, MODY	Osbak (2009) Hum Mutat **30**, 1512
CM096790	CAG-TAG	Gln24Term	c.70C>T	p.Q24*	DM	Diabetes, MODY	Osbak (2009) Hum Mutat **30**, 1512 Xiong (2015) *Science* **347**: 1254806 [Additional report]

PM5: yes

PP1 criterion (Supporting Pathogenicity)

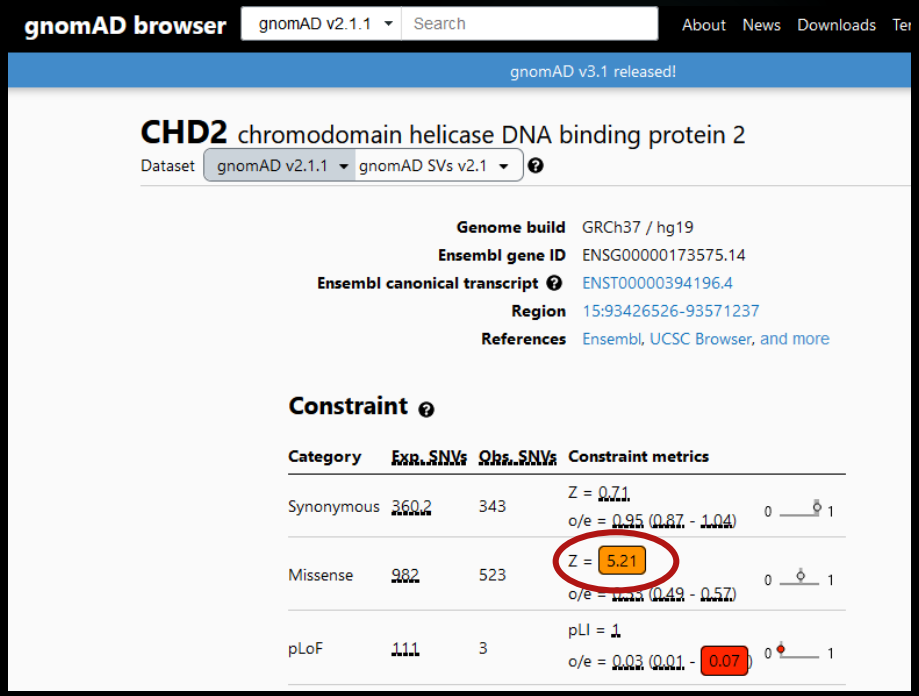
- Cosegregation with disease in multiple affected family members in a gene definitively known to cause the disease
- Needs sequencing of more than three members of the family.
- PP1 = yes if the variant is carried only by ill family's members

PP2 criterion (Supporting Pathogenicity)

- Missense variant in a gene that has a low rate of benign missense variation and in which missense variants are a common mechanism of disease

Where to check:
GnomAD : Constraint function

If Constraint (Missense) Z ≥ 1,75, PP2= yes

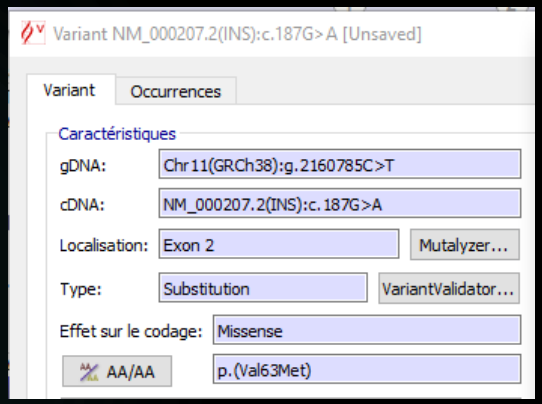


PP3 criterion (Supporting Pathogenicity)

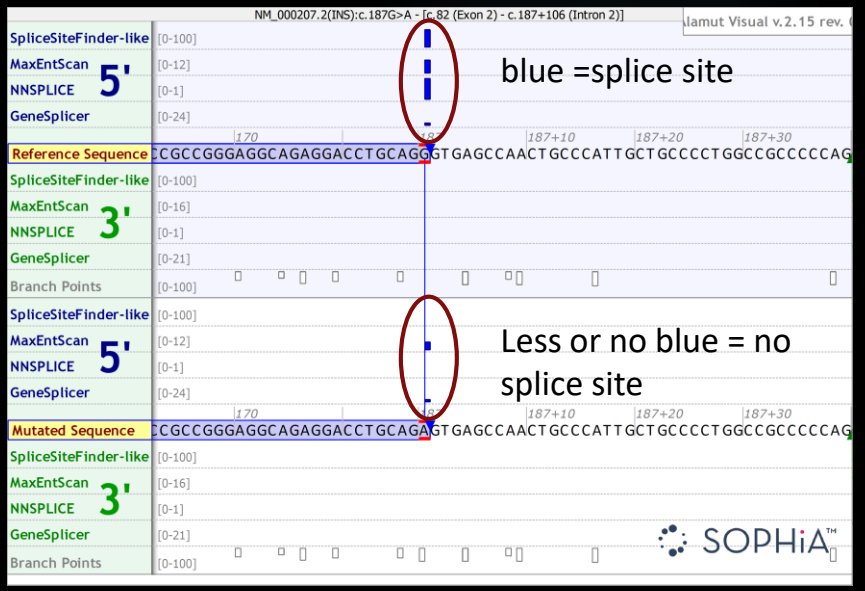
- Multiple lines of computational evidence support a deleterious effect on the gene or gene product (conservation, evolutionary, splicing impact, etc.)

Where to check:
- Alamut
- Splicing predictors

Example: splicing effect:



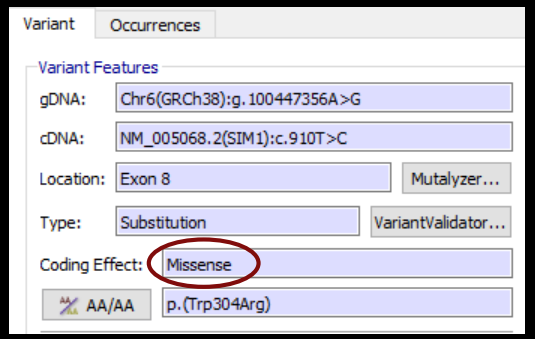
Splicing predictors via Alamut

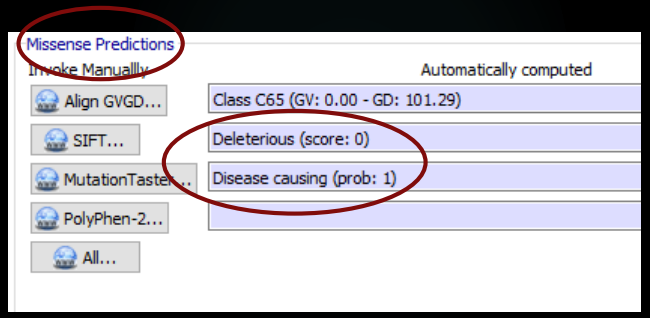


PP3 criterion (Supporting Pathogenicity)

- Multiple lines of computational evidence support a deleterious effect on the gene or gene product (conservation, evolutionary, splicing impact, etc.)

Example: misense





Where to check:

- **Alamut**
- **pathogenicity predictors**

PP3 = yes if : **SIFT: Deleterious**
AND
Mutation Taster: Disease Causing

PP4 criterion (Supporting Pathogenicity)

- **Patient's phenotype or family history is highly specific for a disease with a single genetic etiology**

  ***example: HNf4A, GCK, HNF1A, HNF1B*, … MODY diabetes**

Diagnosis: Rules to classify variants

Table 5 Rules for combining criteria to classify sequence variants

Pathogenic	(i) 1 Very strong (PVS1) *AND* (a) ≥1 Strong (PS1–PS4) *OR* (b) ≥2 Moderate (PM1–PM6) *OR* (c) 1 Moderate (PM1–PM6) and 1 supporting (PP1–PP5) *OR* (d) ≥2 Supporting (PP1–PP5) (ii) ≥2 Strong (PS1–PS4) *OR* (iii) 1 Strong (PS1–PS4) *AND* (a) ≥3 Moderate (PM1–PM6) *OR* (b) 2 Moderate (PM1–PM6) *AND* ≥2 Supporting (PP1–PP5) *OR* (c) 1 Moderate (PM1–PM6) *AND* ≥4 supporting (PP1–PP5)
Likely pathogenic	(i) 1 Very strong (PVS1) *AND* 1 moderate (PM1–PM6) *OR* (ii) 1 Strong (PS1–PS4) *AND* 1–2 moderate (PM1–PM6) *OR* (iii) 1 Strong (PS1–PS4) *AND* ≥2 supporting (PP1–PP5) *OR* (iv) ≥3 Moderate (PM1–PM6) *OR* (v) 2 Moderate (PM1–PM6) *AND* ≥2 supporting (PP1–PP5) *OR* (vi) 1 Moderate (PM1–PM6) *AND* ≥4 supporting (PP1–PP5)
Uncertain significance	(i) Other criteria shown above are not met OR (ii) the criteria for benign and pathogenic are contradictory

Diagnosis: Rules to classify variants

Table 5 Rules for combining criteria to classify sequence variants

Pathogenic	(i) 1 Very strong (PVS1) *AND*
	(a) ≥1 Strong (PS1–PS4) *OR*
	(b) ≥2 Moderate (PM1–PM6) *OR*
	(c) 1 Moderate (PM1–PM6) and 1 supporting (PP1–PP5) *OR*
	(d) ≥2 Supporting (PP1–PP5)
	(ii) ≥2 Strong (PS1–PS4) *OR*
	(iii) 1 Strong (PS1–PS4) *AND*
	(a)≥3 Moderate (PM1–PM6) *OR*
	(b)2 Moderate (PM1–PM6) *AND* ≥2 Supporting (PP1–PP5) *OR*
	(c)1 Moderate (PM1–PM6) *AND* ≥4 supporting (PP1–PP5)

Example: selected criteria are:

- **PVS1, PM1, PP2 : the variant is pathogenic**
- **PS1, PS3, PM2: the variant is pathogenic**
- **PVS1: the variant is VUS (Variant Uncertain Significance)**

Diagnosis: Rules to classify variants

Likely pathogenic	(i) 1 Very strong (PVS1) *AND* 1 moderate (PM1–PM6) *OR* (ii) 1 Strong (PS1–PS4) *AND* 1–2 moderate (PM1–PM6) *OR* (iii) 1 Strong (PS1–PS4) *AND* ≥2 supporting (PP1–PP5) *OR* (iv) ≥3 Moderate (PM1–PM6) *OR* (v) 2 Moderate (PM1–PM6) *AND* ≥2 supporting (PP1–PP5) *OR* (vi) 1 Moderate (PM1–PM6) *AND* ≥4 supporting (PP1–PP5)
Uncertain significance	(i) Other criteria shown above are not met OR (ii) the criteria for benign and pathogenic are contradictory

Example: selected criteria are:

- **PVS1, PM1: the variant is likely pathogenic**
- **PS1, PM2, PM5: the variant is likely pathogenic**
- **PM2, PP2, PP3, PP4: the variant is VUS (Variant Uncertain Signifiance)**
- **PVS1 only: variant VUS**