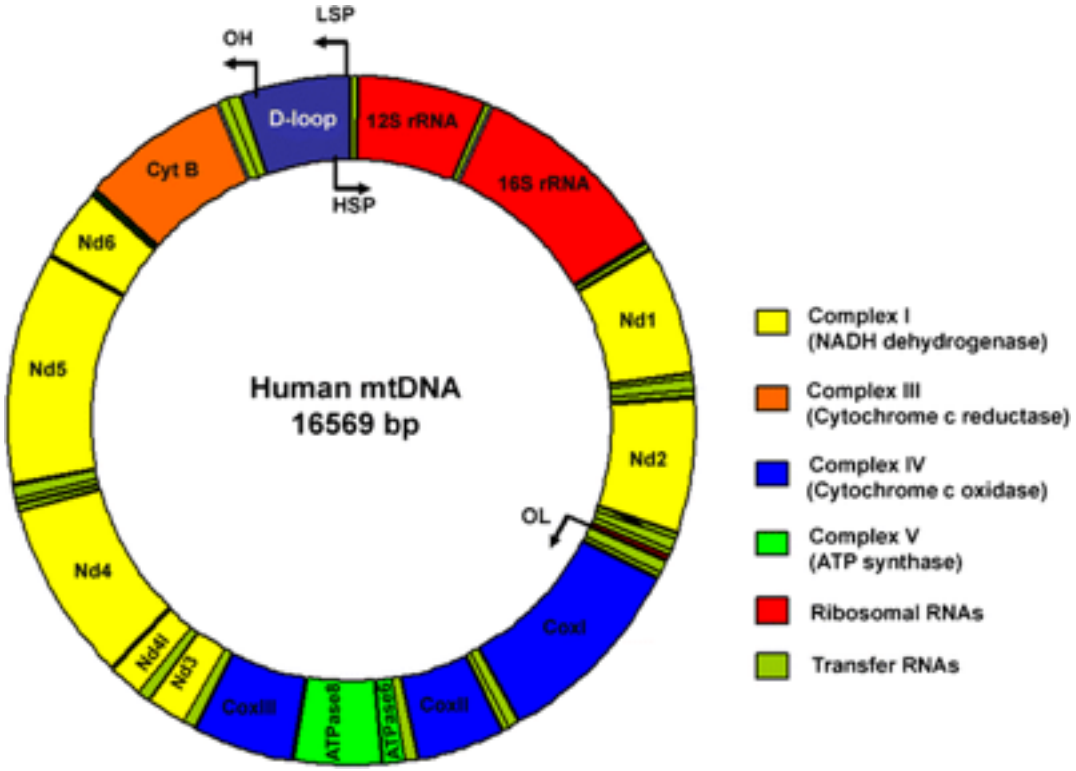
Mitochondria and mitochondrial DNA (mtDNA)

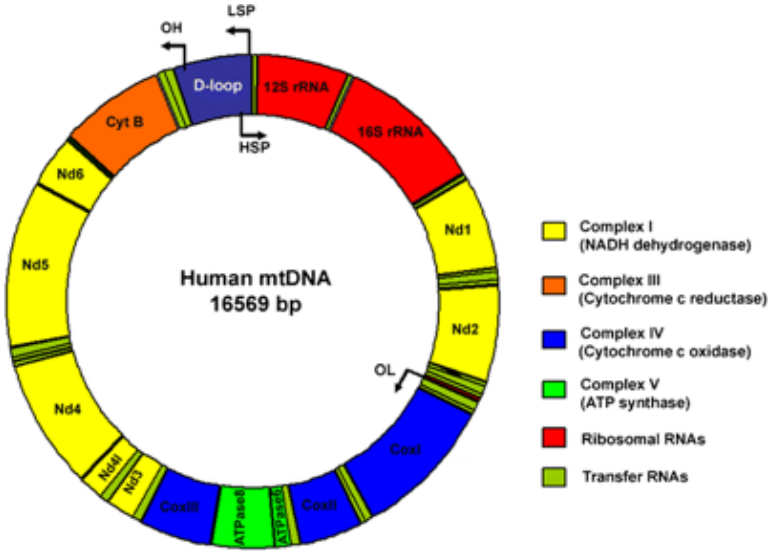
The mitochondrion is a **highly specialized organelle**, present in almost all eukaryotic cells and principally charged with the production of **cellular energy through oxidative phosphorylation (OXPHOS)**.

Mitochondria are also involved in calcium signalling, regulation of cellular metabolism, haem synthesis, steroid synthesis and, perhaps most importantly, programmed cell death (apoptosis).

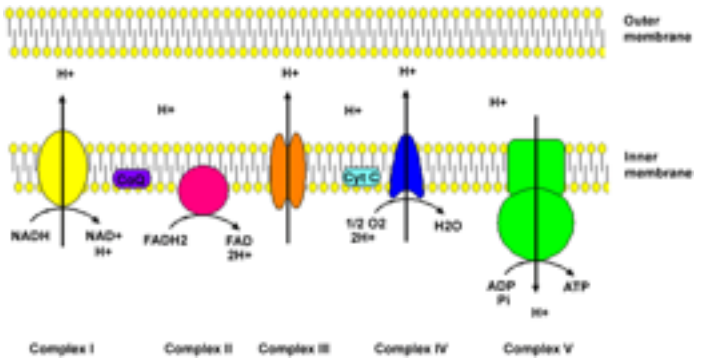


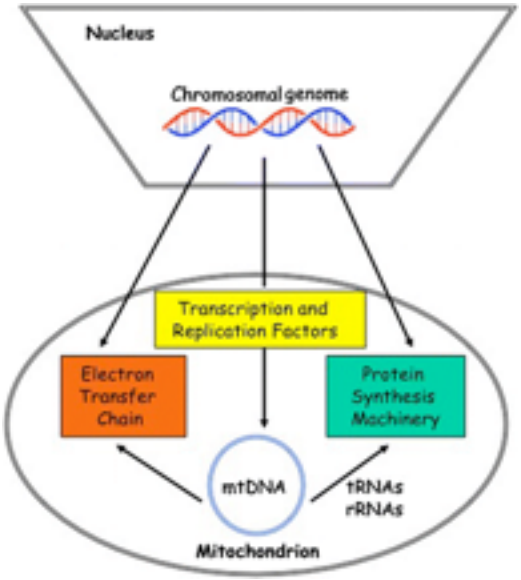
- Mitochondria have their own DNA, known as mitochondrial DNA or **mtDNA**.
- In humans, mitochondrial DNA spans about 16,500 DNA base pairs, representing a small fraction of the total DNA in cells.

Mitochondria and mitochondrial DNA (mtDNA)



MtDNA consists in a heavy (H) and a light (L) strand, which encodes **13** of the more than 90 subunits of the electron-transfer chain and 22 tRNAs and two rRNAs.

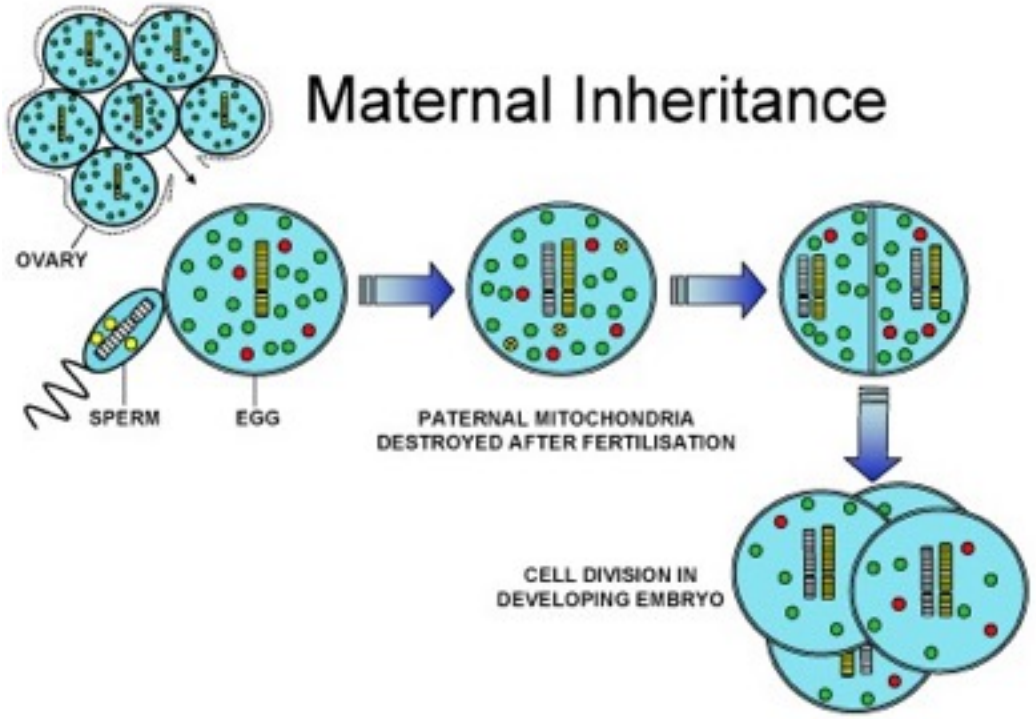




Mitochondria divide by **binary fission**, similar to bacterial cell

In mammals mitochondria may replicate their DNA and divide mainly **in response to the energy needs of the cell**, rather than in phase with the cell cycle

Mammals normally inherit their mtDNA from the population present in the oocyte, just prior to fertilization when mtDNA replication has been completed (**matrilinear inheritance**)



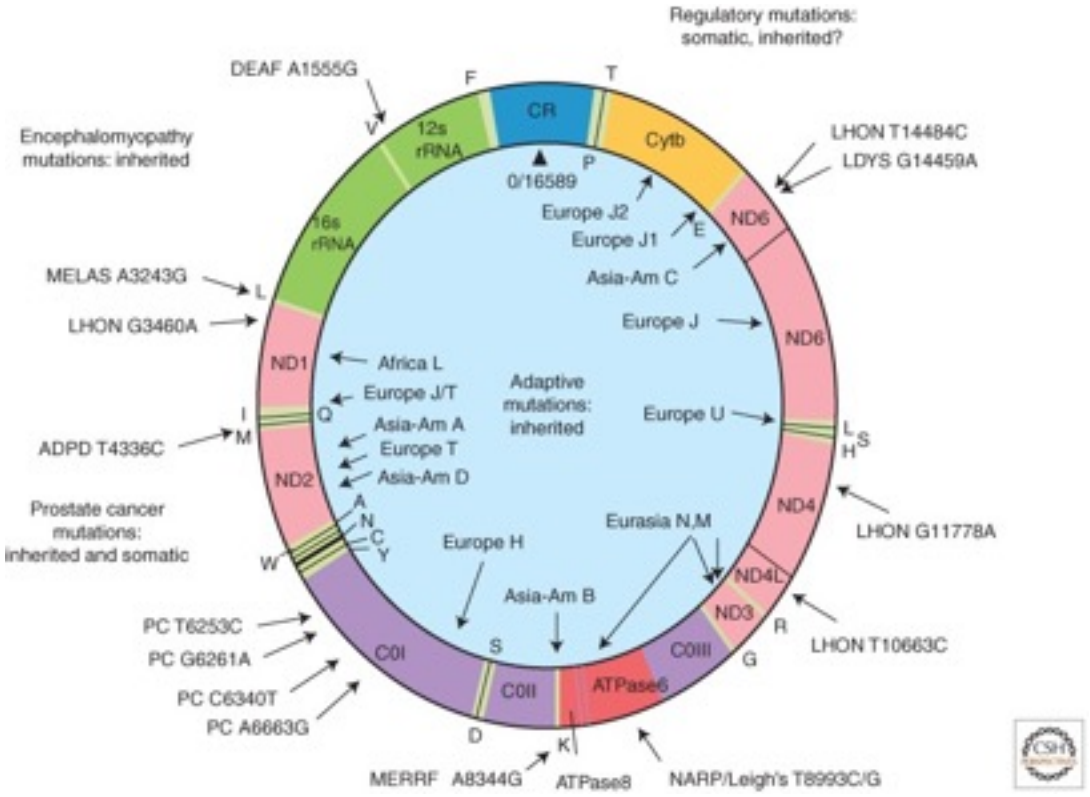
Uniparental inheritance leads to little opportunity for genetic recombination between different lineages of mitochondria

These copies tend to be identical and thus the mtDNA transmitted to the offspring is **homoplasmic**.

Following natural fertilization, sperm mtDNA tends to be eliminated thus ensuring the maintenance of homoplasmy

The **near-absence of genetic recombination** in mitochondrial DNA makes it a useful source of information for population genetics and evolutionary biology.

Mitochondrial DNA is inherited **as a single unit**, or haplotype that can be used to infer the evolutionary history of populations.

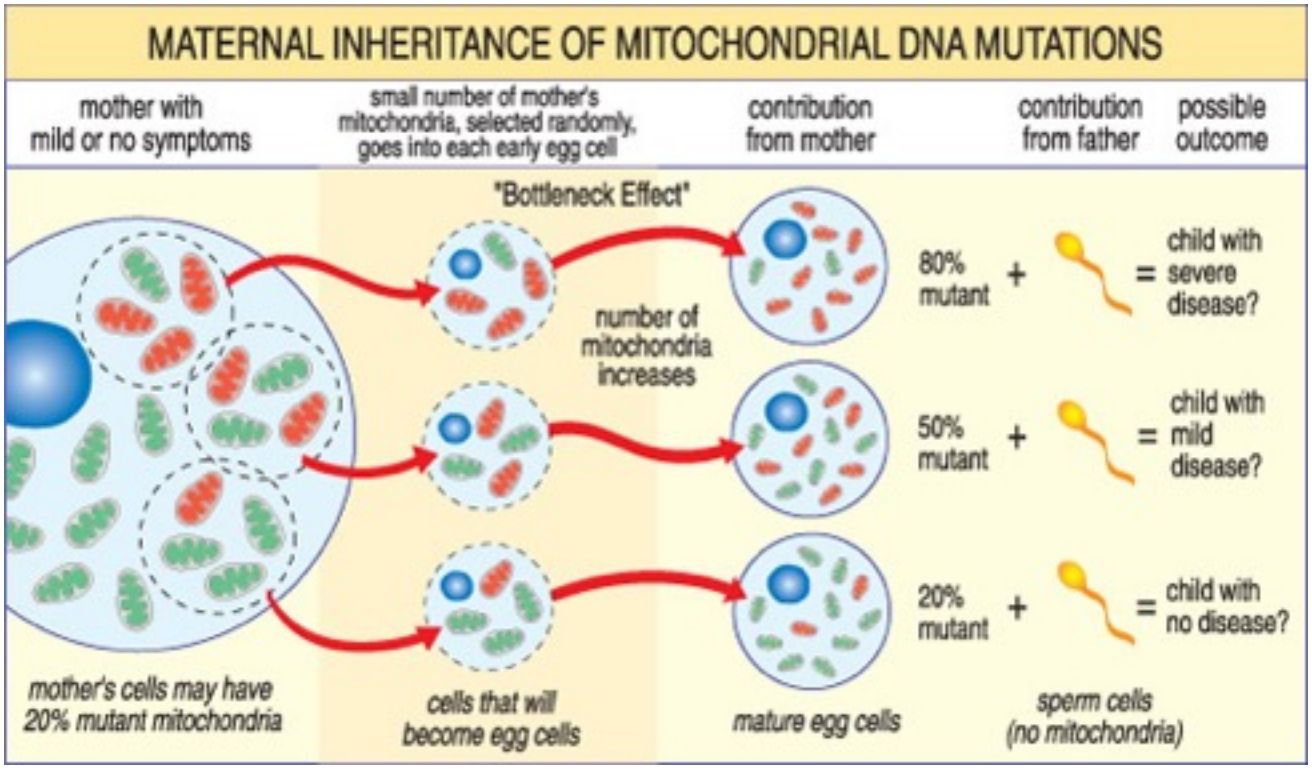


The segregation of mtDNA molecules tends to follow a pattern of **random genetic drift since variants can be fixed (heteroplasmic condition)**

Normally, all of the thousands of copies of mtDNA within an individual are wild-type (WT) and identical, i.e. homoplasmic.

However, **mutant and WT mtDNA molecules can coexist** in a state described as **heteroplasmy**.

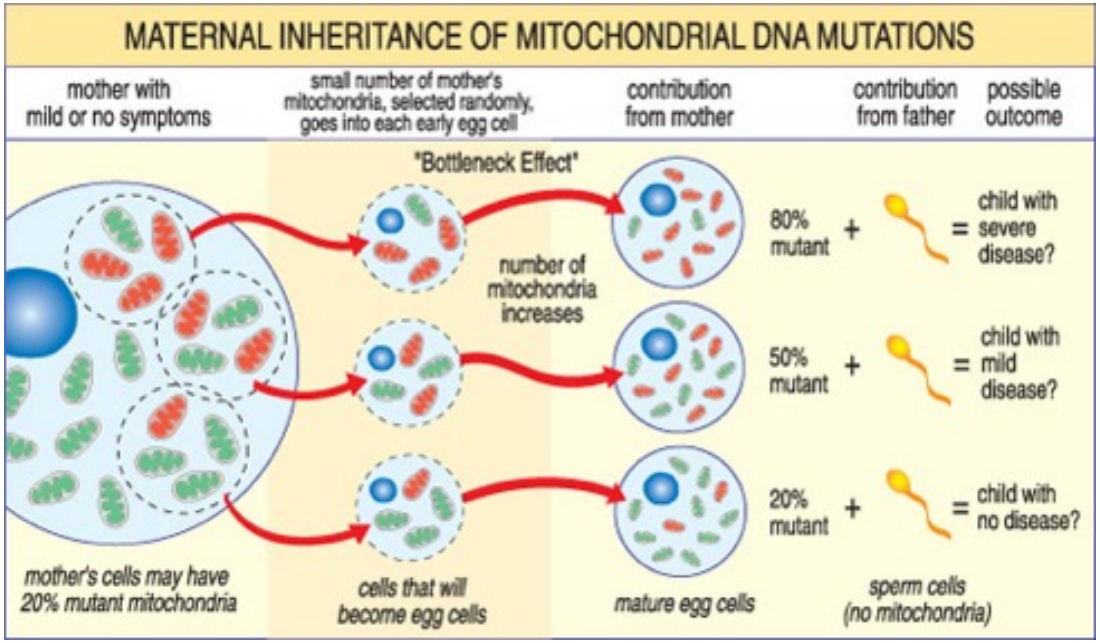
Over 100 point mutations and large-scale deletions have been identified so far



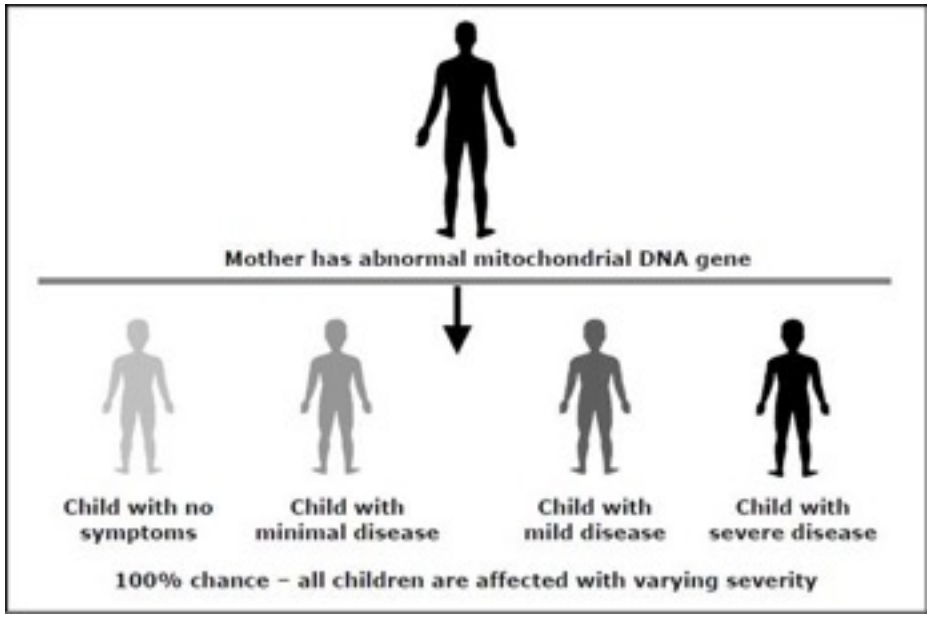
1 in 10,000 of the population are affected by **mtDNA disease** and another 1 in 6000 are at risk

Mitochondria, undergoing **uniparental inheritance** and with **little to no recombination** should accumulate deleterious mutations until functionality is lost.

Mitochondria avoid this buildup through a developmental process known as the **mtDNA bottleneck:** a single egg cell with some proportion of mutant mtDNA produces an embryo where different cells have different mutant loads



Cell-level selection may then remove those cells with more mutant mtDNA, leading to a stabilisation or reduction in mutant load between generations.



- Mitochondrial disease is an **inherited chronic illness** that can be present at birth or develop later in life.
- It causes **debilitating physical** and **cognitive disabilities**, loss of muscle coordination; muscle weakness and pain; seizures; vision and/or hearing loss; learning disabilities

- mtDNA inherited diseases affect **many tissues with variable features**.
- there are hundreds of different mitochondrial diseases with a **spectrum of abnormalities**
- identical mtDNA mutations may not produce identical diseases.

Inherited mitochondrial diseases show complex patterns of inheritance

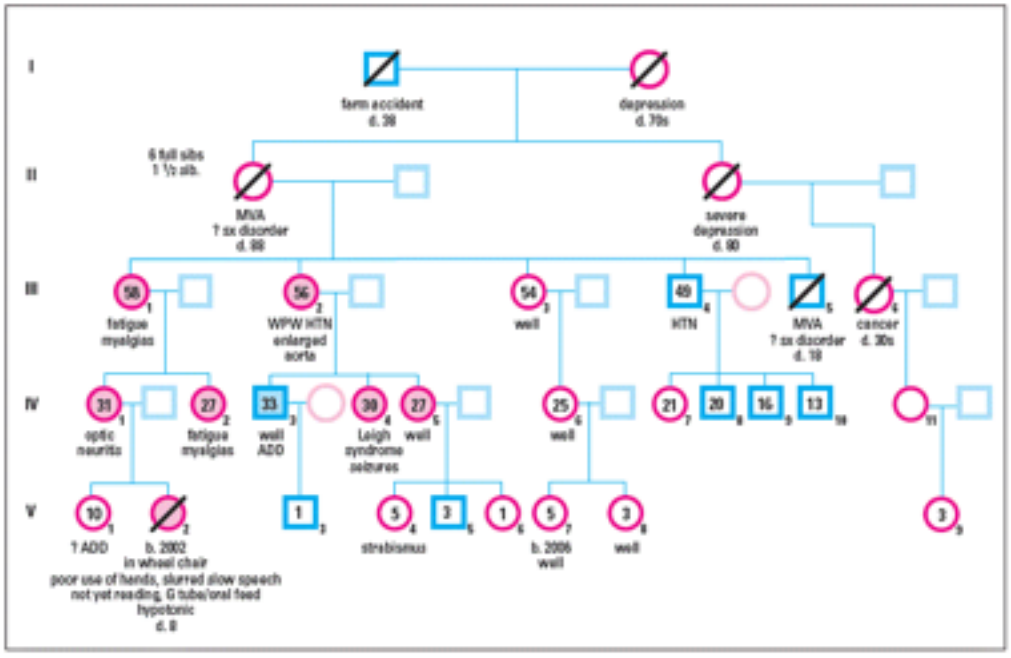
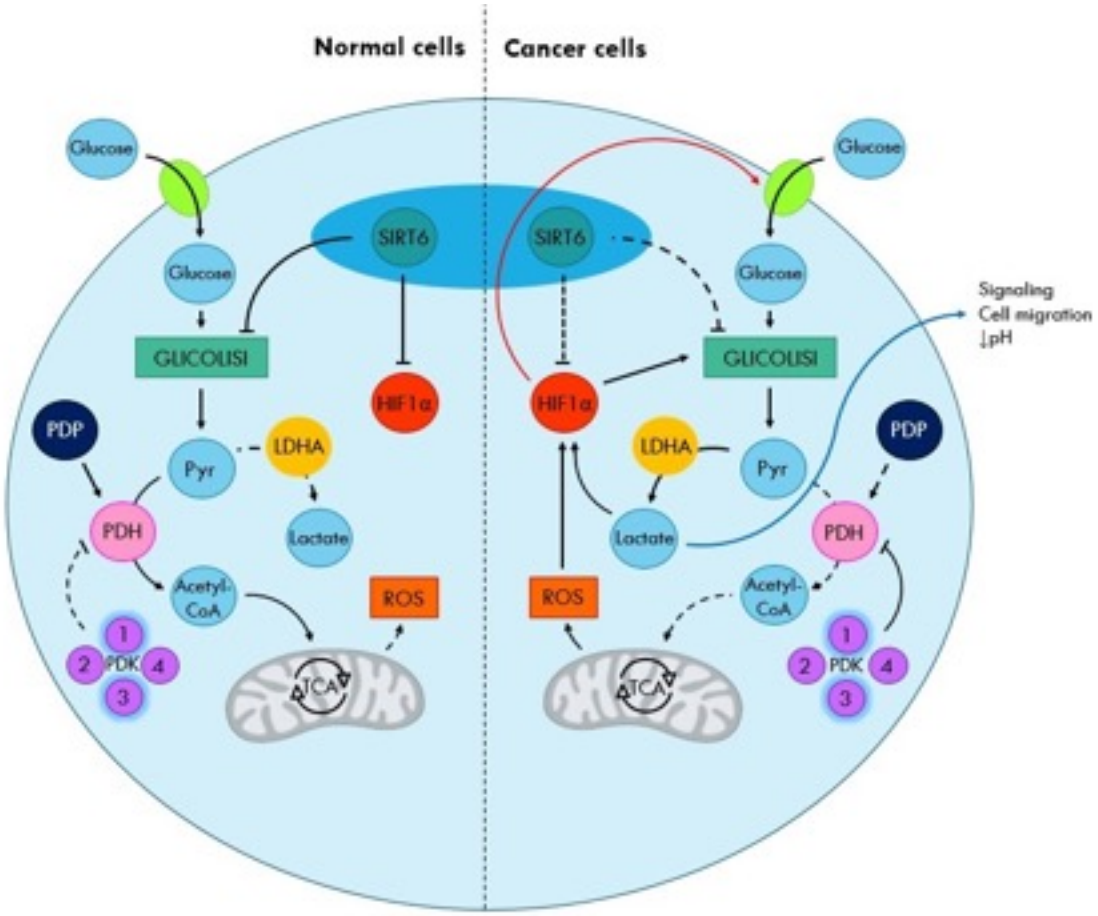


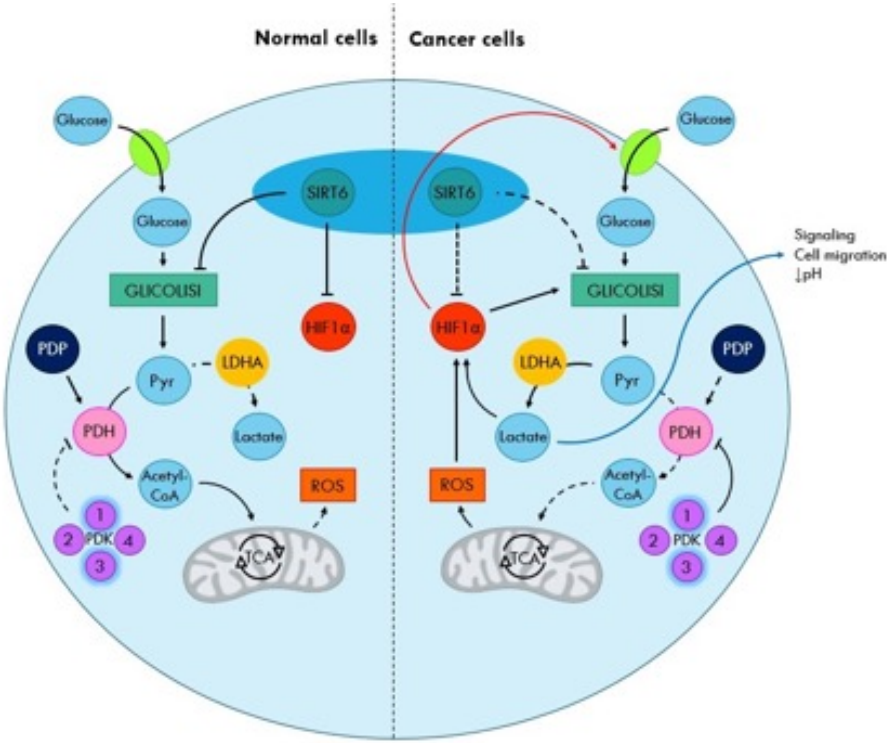
Figure 1. Family A pedigree. A familial pathogenic mutation in mitochondrial DNA was first identified in patient IV-4. Affected members of this large EC family exhibit symptoms ranging from mild to severe.



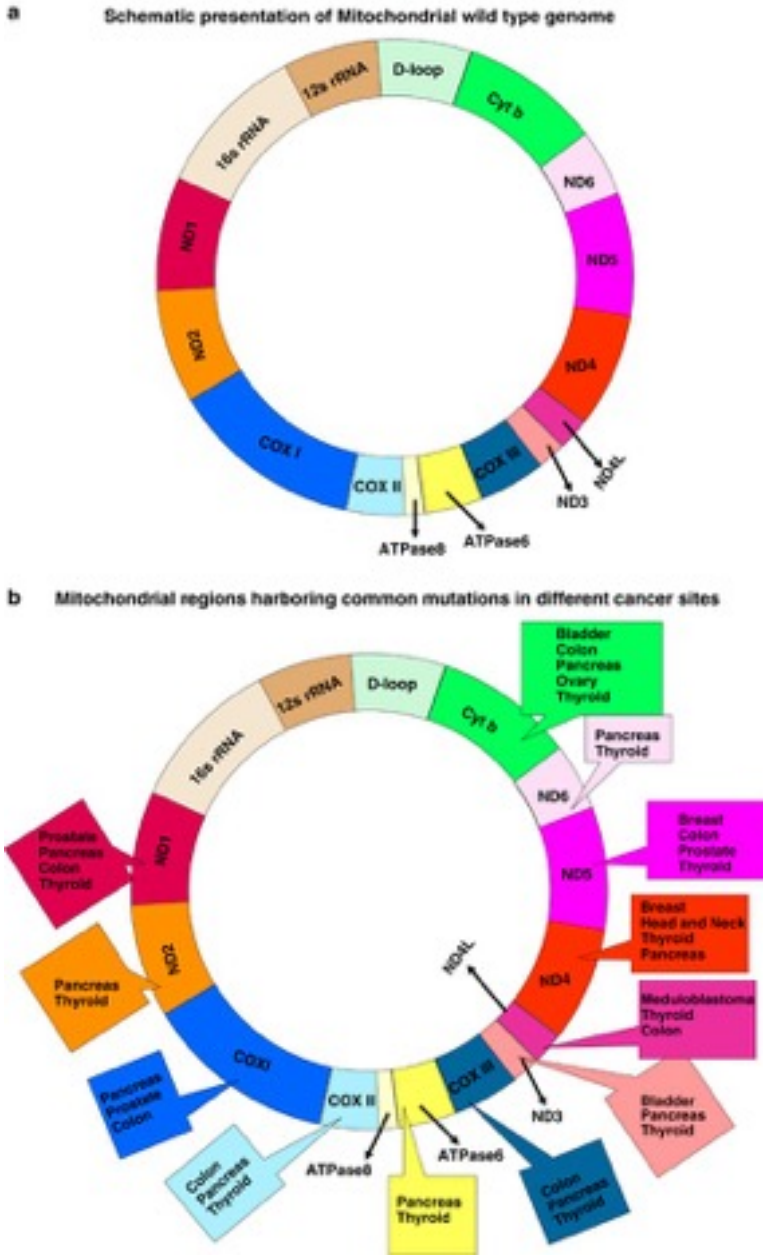
- Cancer cells are metabolically adapted for rapid growth and proliferation under hypoxic conditions, a condition in which normal cells would not grow at all or only poorly
- **Metabolic switch : glicolisi increase**

- **Altered metabolism in cancer cells** has been directly or indirectly linked to mitochondria

- Differences in the **ultra structure** of mitochondria and **depletion cellular mitochondrial numbers** have been reported in cancer cells



- Differences in **content and composition** of all oxidative phosphorylation complexes, respiratory chain activity, expression of oxidative phosphorylation genes and levels of mitochondrial DNA were reported relative to normal controls.



High level of ROS

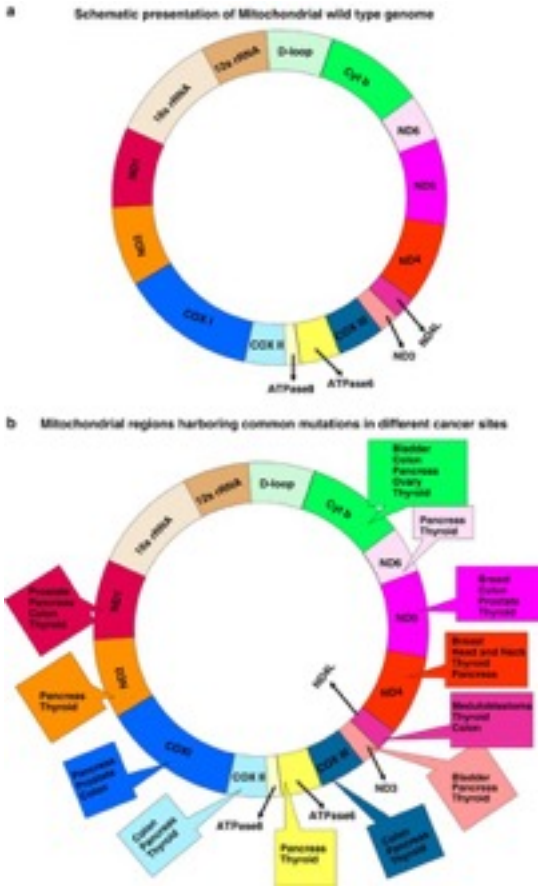
↓

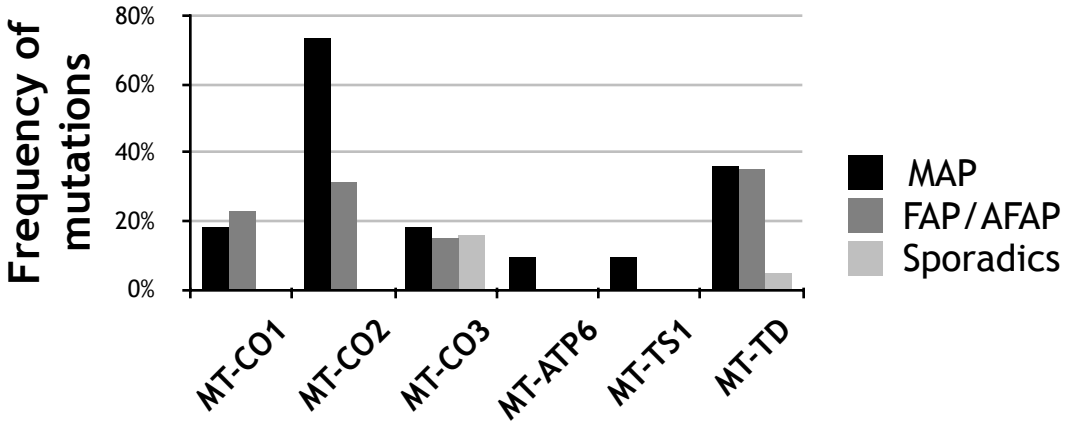
Several mtDNA mutations have been identified in **various types of human cancer**.

Mutations have been found in both the **non-coding region and coding regions of the mtDNA**

The majority of the mutations appeared to be **homoplasmic** in nature

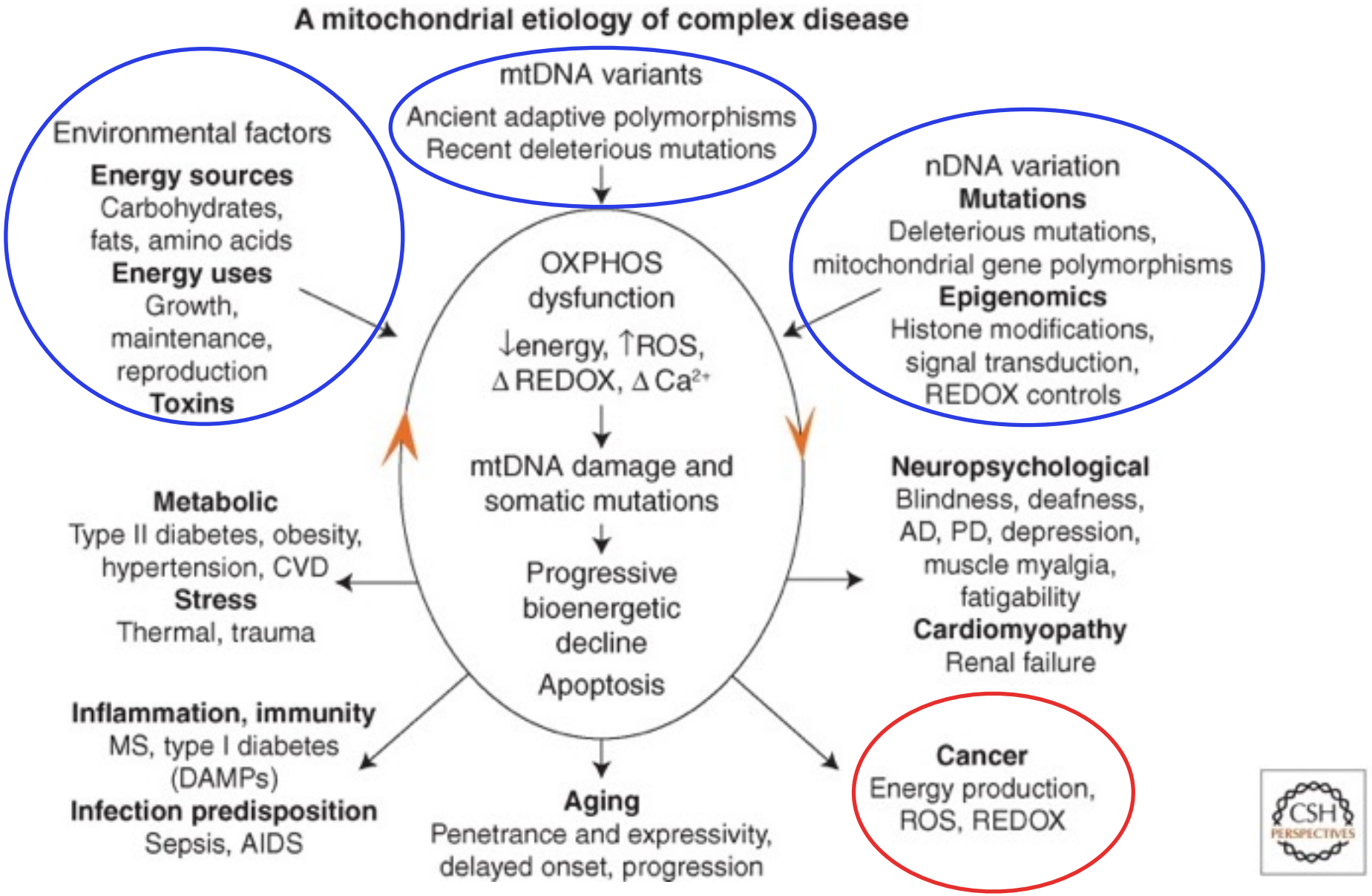
Specific mutations in **"target" mitochondrial genes** of MAP adenomas and carcinomas



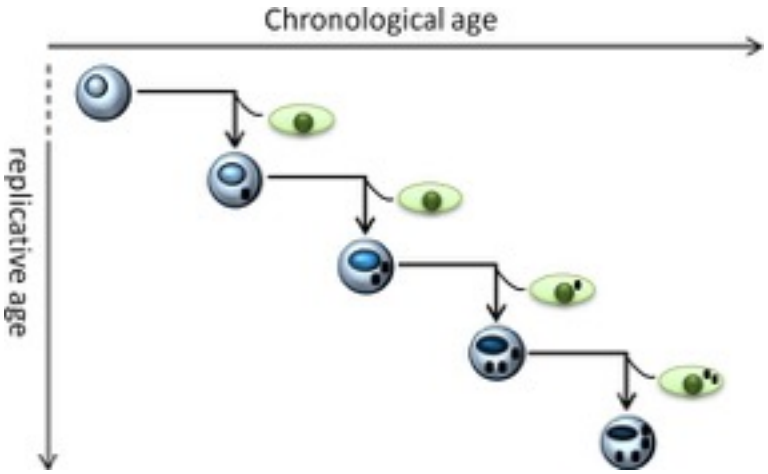


Errichiello et al., 2015

…The sequence analysis revealed **17 different variants**, in the **MT-CO2** gene of MAP patients (P<0.0001) who frequently carried the hotspot m.7763 G>A mutation…**D-loop instability** was also significantly associated with variants grouped inside the MT-CO2 gene (P=0.006)…


A mitochondrial etiology of complex disease
mtDNA variants
Ancient adaptive polymorphisms
Recent deleterious mutations
Environmental factors
Energy sources
Carbohydrates, fats, amino acids
Energy uses
Growth, maintenance, reproduction
Toxins
nDNA variation
Mutations
Deleterious mutations, mitochondrial gene polymorphisms
Epigenomics
Histone modifications, signal transduction, REDOX controls
OXPHOS dysfunction
↓energy, ↑ROS, Δ REDOX, Δ Ca2+
mtDNA damage and somatic mutations
Progressive bioenergetic decline
Apoptosis
Metabolic
Type II diabetes, obesity, hypertension, CVD
Stress
Thermal, trauma
Neuropsychological
Blindness, deafness, AD, PD, depression, muscle myalgia, fatigability
Cardiomyopathy
Renal failure
Inflammation, immunity
MS, type I diabetes (DAMPs)
Infection predisposition
Sepsis, AIDS
Aging
Penetrance and expressivity, delayed onset, progression
Cancer
Energy production, ROS, REDOX

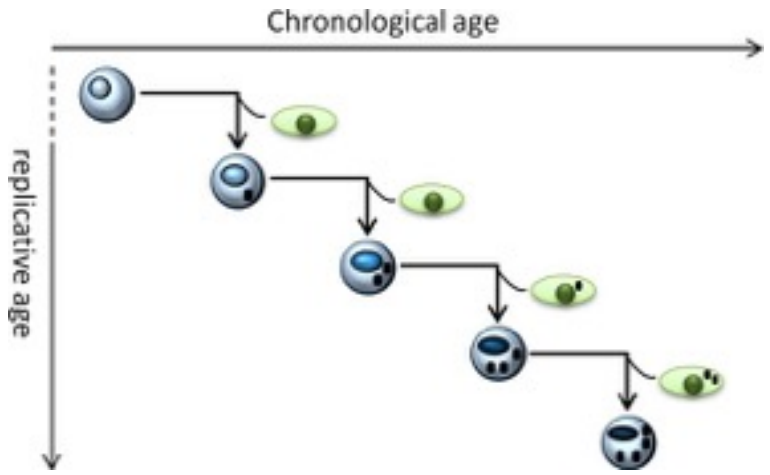
STEM CELLS AND CANCER



Adult stem cells (SCs) maintain tissue homeostasis throughout life and are **rare, largely quiescent cells** capable of :

- **self-renewal**
- **maintaining the stem cell pool**
- **differentiating** to produce all mature cells within a tissue

STEM CELLS AND CANCER

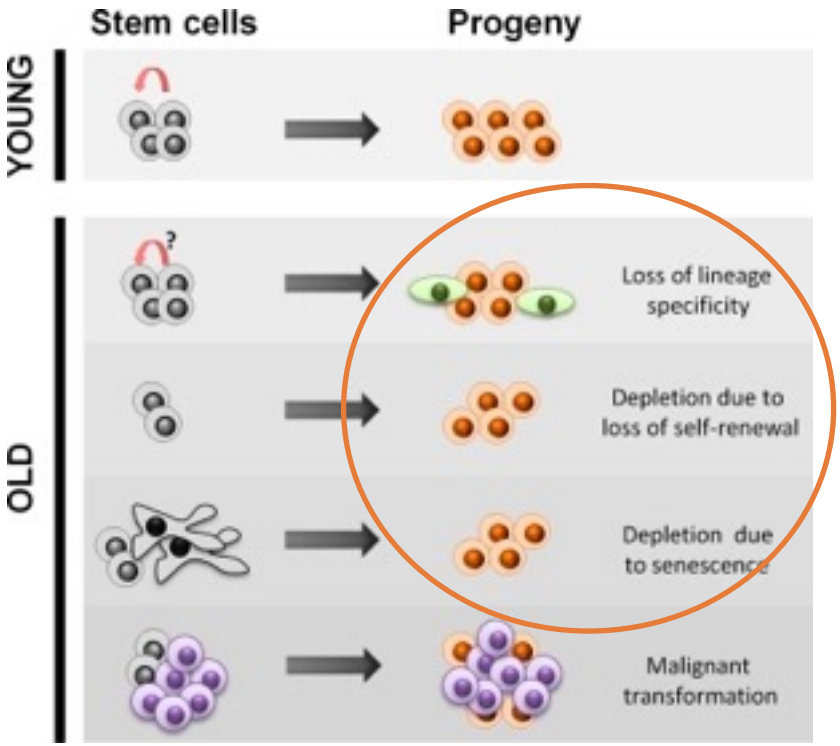


Asymmetric cell division allows SCs to 1) **self-renew** and 2) produce **another cell** that undergoes differentiation, providing tissue homeostasis.

The **SC daughter(s)** of a stem cell retain **all stem cell characteristics:**
1) ***proliferation capacity,***
2) undifferentiated state,
3) the capability to produce daughter cells that undergo differentiation.

A **failure to maintain the correct stem cell number** leads to **tumorigenesis/ tissue hyperplasia** by stem cell hyperproliferation or **tissue degeneration/ aging**

STEM CELLS AND CANCER



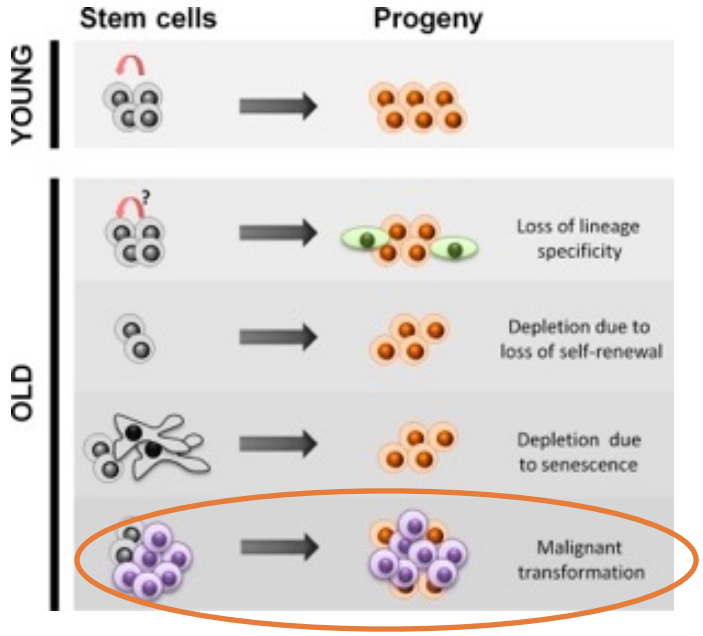
Wnt, Notch and Hedgehog pathways are the controllers of the balance between **self-renewal and differentiation** in *neural, epidermal, intestinal, breast and haematopoietic SCs*.

Cell survival and cell-cycle-regulating pathways, such as **p53, Bmi-1 and cyclin-dependent kinase inhibitors (CDKI)**, represent an additional intrinsic regulatory mode of SC self-renewal.

Also SCs are subjected to aging by accumulating DNA or protein damages

Aging induces the **lost of specificity and and functionality** with the onset of symmetric divisions.

STEM CELLS AND CANCER



SCs have the ability to self-renew and differentiate to give rise to mixed cellular populations.

These features suggested that **tumour-initiating cells** might in fact be **cancer stem cells**.

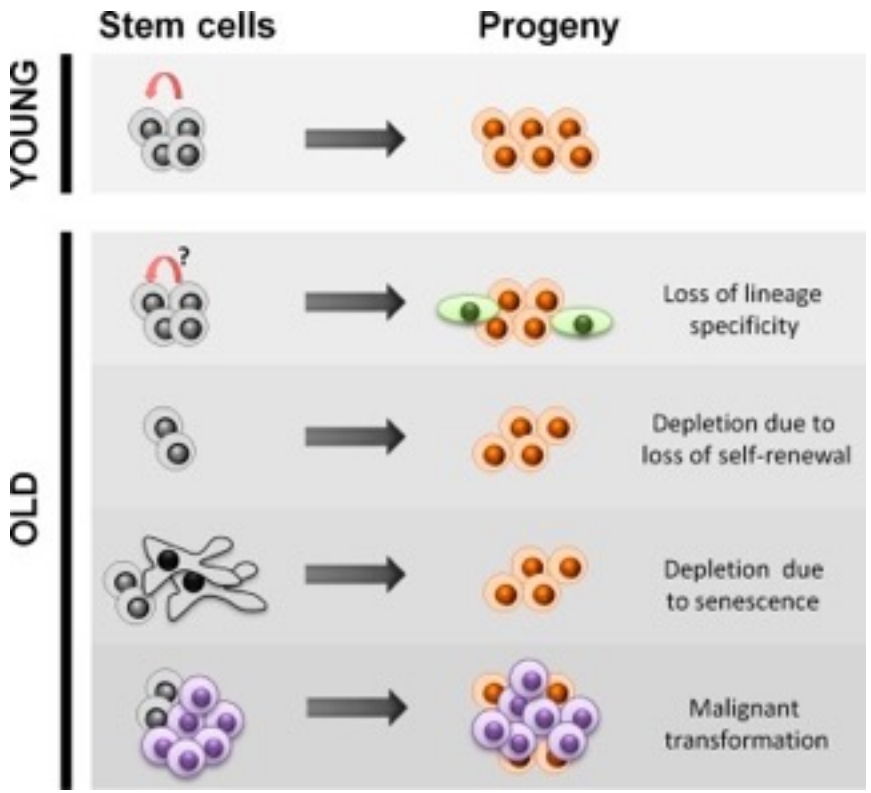
Cancer stem cells could explain the **heterogeneity** of most tumours and the **relapse** occurring in patients considered tumour-free for many years.

The existence of CSCs, and whether they are sufficient to maintain tumour growth in humans, has not yet been definitively confirmed.

It is still unclear whether tumour initiation is driven by :

1) ***genomically advantaged stem cells*** *or*
2) ***more differentiated cells that reacquire stem cell properties***
3) *both events are possible*

STEM CELLS AND CANCER



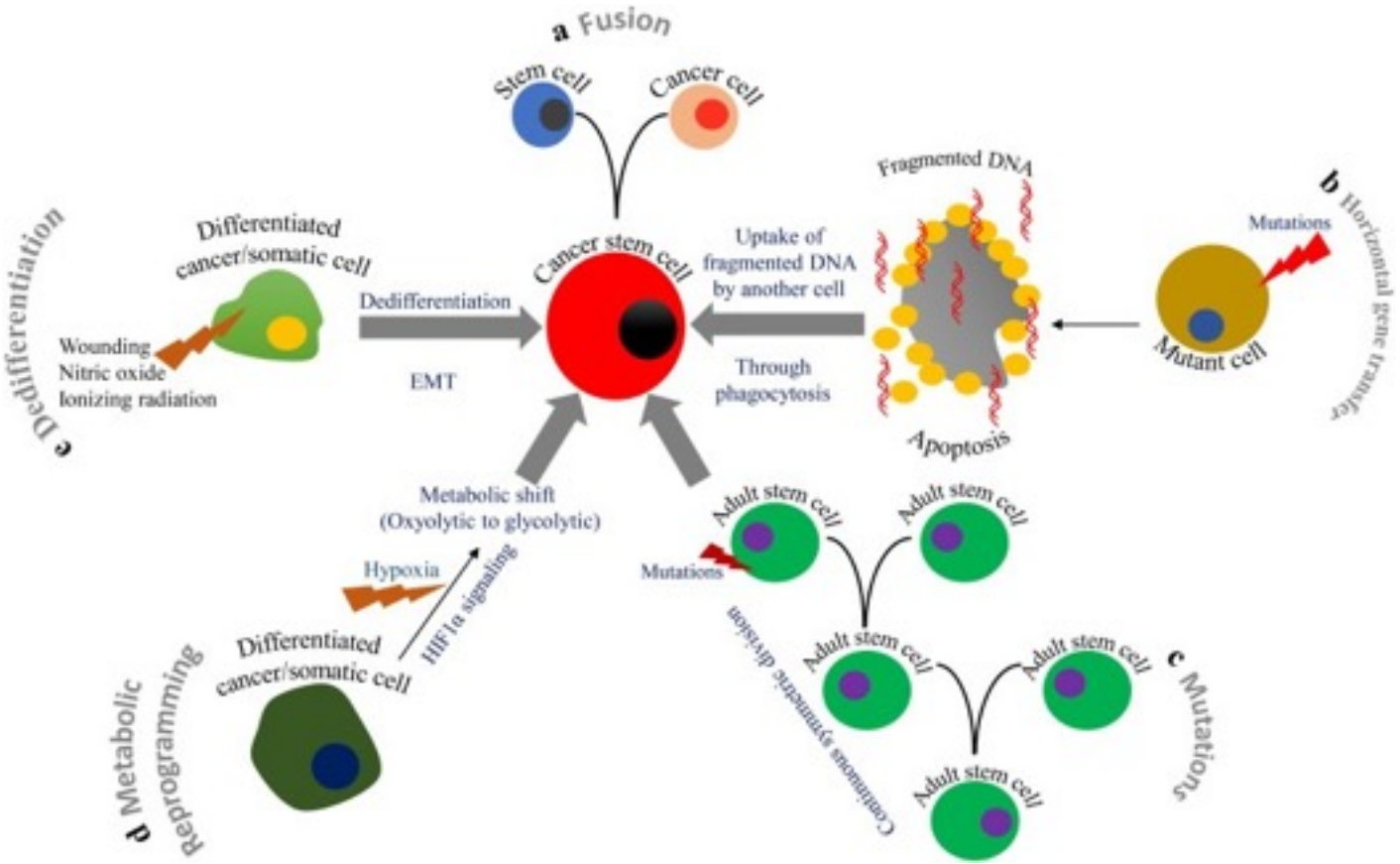
A <u>tight control on SC asymmetric cell divisions</u> is important to prevent the formation of aberrant SC pools with unrestrained proliferation, which might result in overgrowing tissues.

*CSCs are originated by **<u>cell fusion</u>** , **<u>horizontal gene transfer</u>**, **<u>mutations</u>** in <u>somatic</u> or differentiated <u>cancer cells</u>, resulting in de-differentiation and reprogramming.*

Recent studies also provided evidence for the existence of distinct or heterogeneous CSC populations within a single heterogeneous tumor

Cancer stem cells (CSCs) can self-renew by dividing and giving rise to many cell types that constitute the tumor.

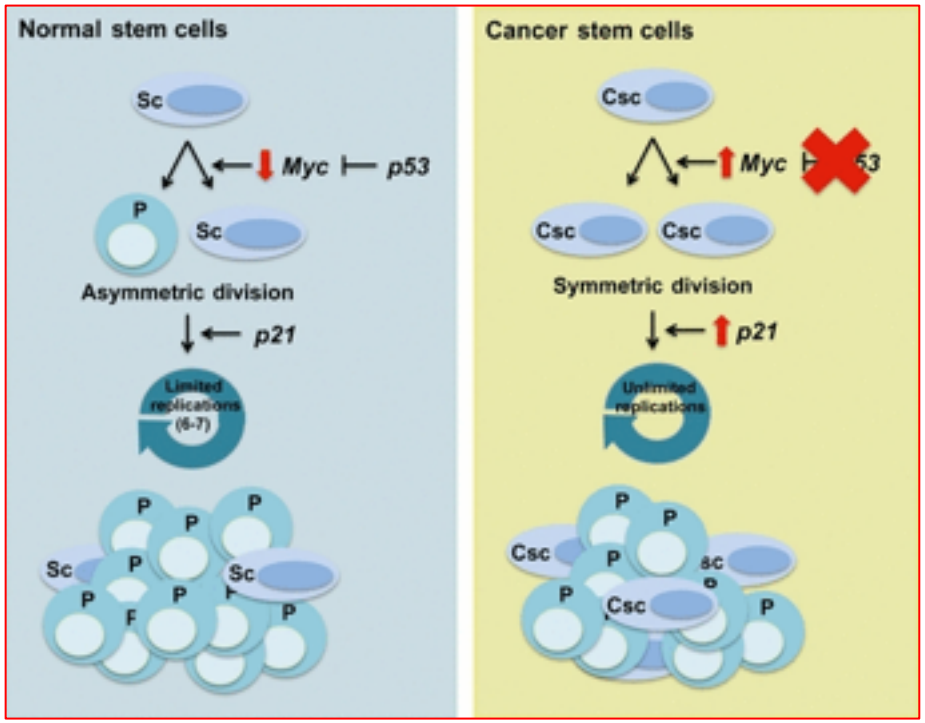
CSCs also have been shown to be involved in fundamental processes of cell proliferation and metastatic dissemination.



Stem cells can be target of transforming mutations, as demonstrated for certain leukaemias, or acquires a gain of function mutation that endows it with self-renewal capability

STEM CELLS AND CANCER

Verga Falzacappa FEBS Journal 279 (2012) 3559–3572



*Normal SCs divide **mainly asymmetrically** giving rise to stem (Sc) and progenitor (P) cells*

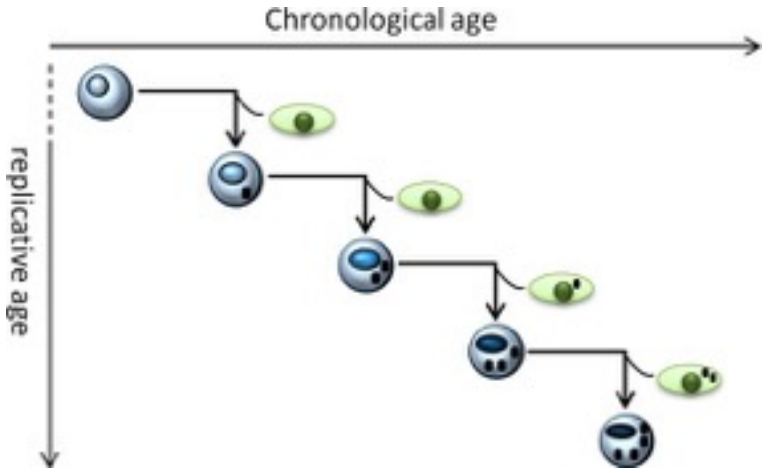
Their self-renewal potential is **intrinsically restricted**, exhausting when they reach the limit of **six to seven divisions**

In normal SCs, **p53-dependent regulation of c-Myc imposes an asymmetric mode** of division and **p21 maintains self- renewal**

- In **cancer stem cells (Csc)**, self-renewal capability is deregulated.
- **Loss of p53 results in a switch to the symmetric mode of cell division**, and upregulation of p21 extends the self-renewal ability of CSCs.
- The **CSCs undergo an indefinite number of rounds of cell division**, which results in the expansion of the stem cell pool.

STEM CELLS AND CANCER

*SCs are equipped with specific and effective **DNA-damage response mechanisms** to avoid propagation of genetic lesions to all their progeny.*



*SCs have specific **transporters** that **pumps genotoxic compounds out of the cells** and they are mostly metabolically inactive, minimizing replication errors and the production of ROS*

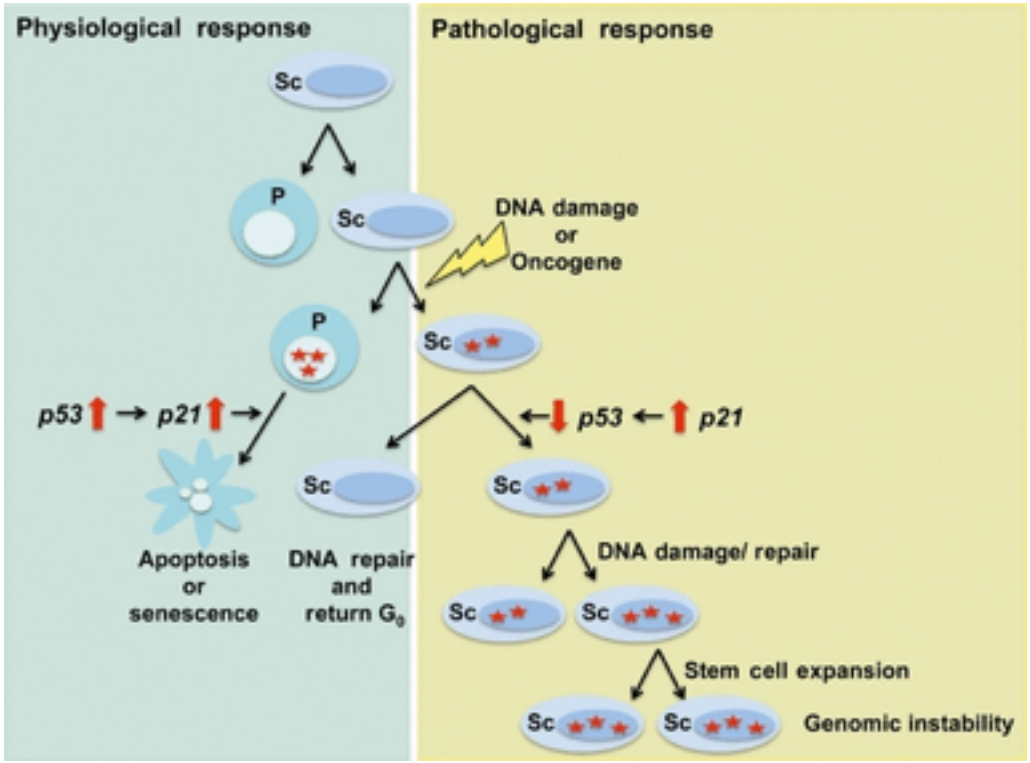
They are subjected to DNA damage

In most adult tissues SCs appear to be more resistant to DNA damage than their differentiated progeny

SCs survive by activation of specific prosurvival and DNA-repair responses

***Upregulation of p53** in all these type of cells has been observed during these responses to **induced DNA damage**, irrespective of their degree of differentiation*

STEM CELLS AND CANCER



The DNA-damage response is different between stem (Sc) and progenitor (P) cells.

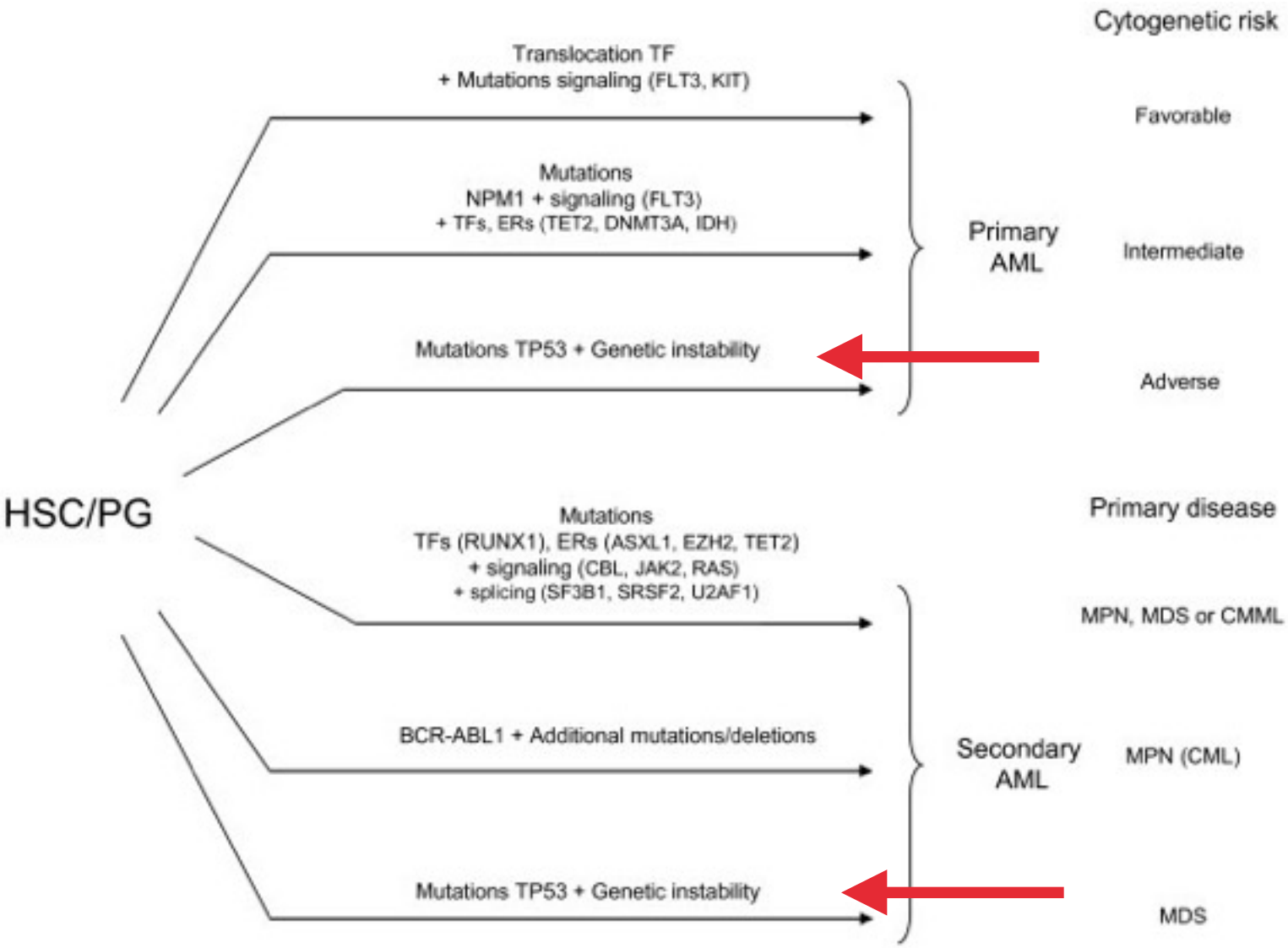
- ***Progenitor cells*** *respond to damage via* ***p53-dependent upregulation of p21*** *that induces apoptosis or senescence*

- ***SCs upregulate p21, resulting in downregulation of p53 activity****, which inactivates apoptotic responses and increases* ***cell cycle entry and expansion of the SC pool,*** *increasing the rounds of symmetric divisions.*

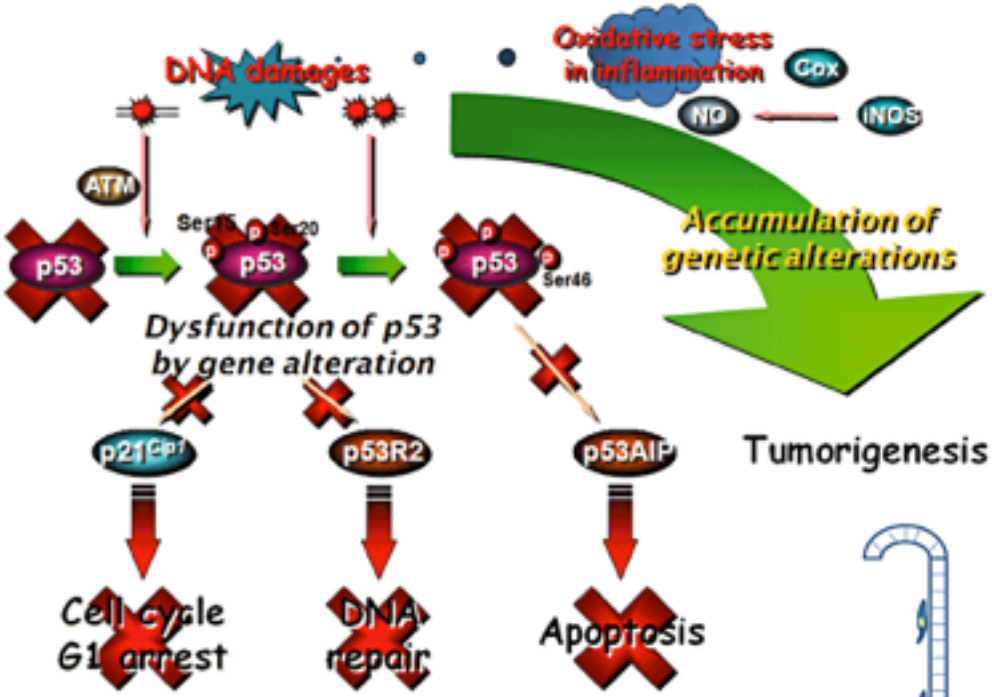
Continuous DNA damage and repair suppresses apoptosis/senescence favouring the survival of SCs that harbour DNA mutations.

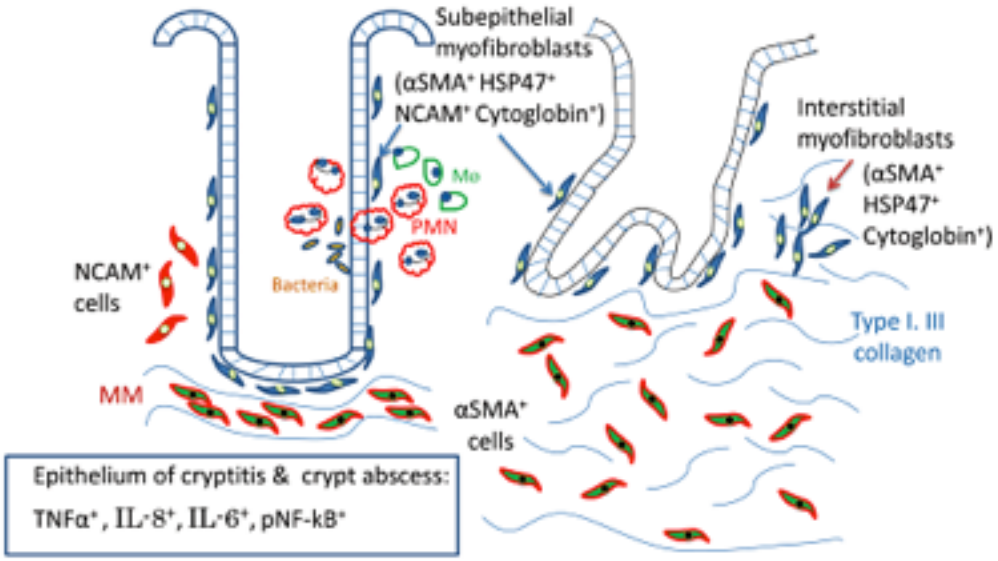
This could generate an actively expanding pool of **immortal and genomically unstable SCs increasing the risk of cancer**.

STEM CELLS AND HEMATOPOIETIC CANCER

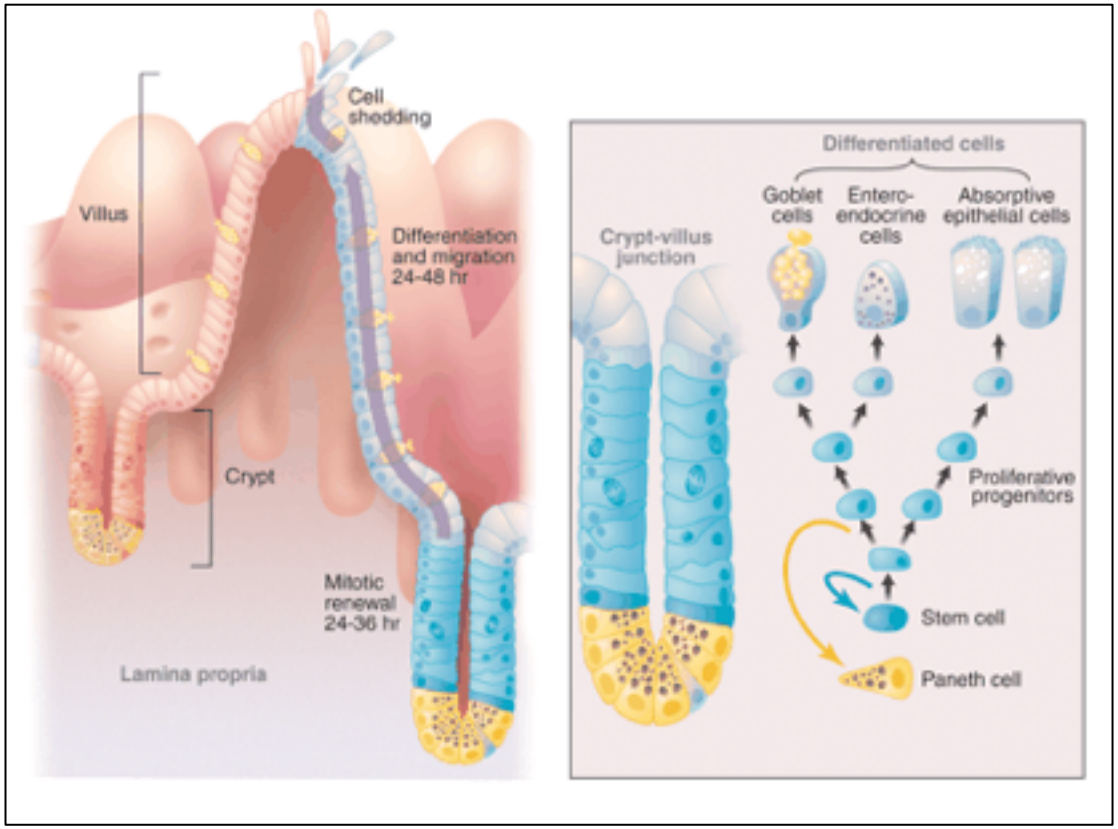


STEM CELLS AND GASTRIC CANCER





STEM CELLS AND COLORECTAL CANCER

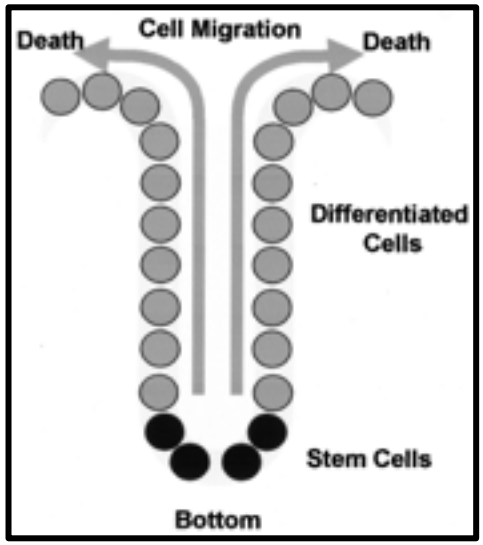


Human colon is composed of 5×10^6

1/6- 1/3 of these cells fell into intestinal lumen every 24 hours

Genes involved in this regulation are important in the first steps of tumorigenesis

- Stem cells are located in the **stem cell niche** at the bottom of the crypt, among Paneth cells, and are responsible for the maintenance of **crypt homeostasis**.
- They were first investigated by Cheng and Leblond, who called them **"crypt base columnar cells."**

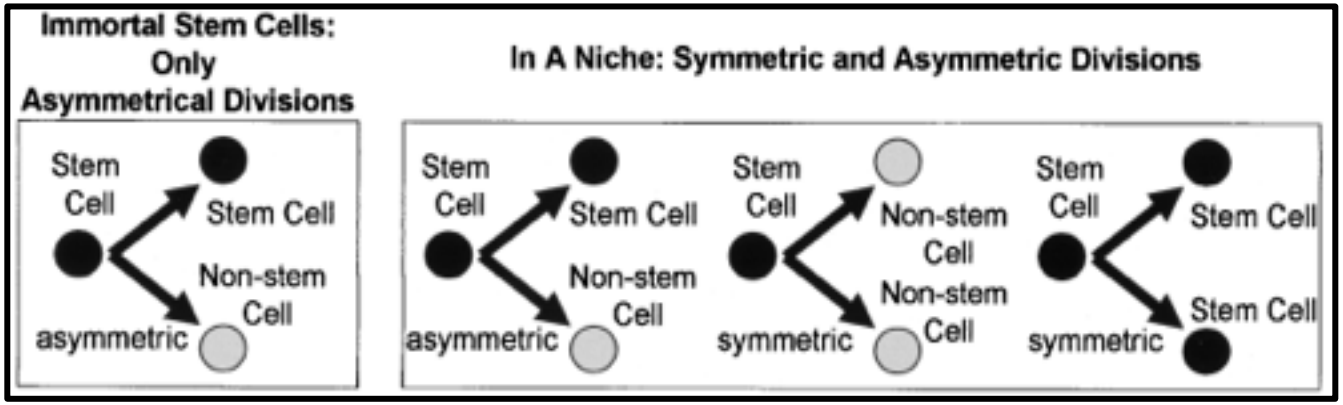


The complete life cycle of these cells takes about **5 days**, and **the entire epithelial lining of the gut is replaced once a week**

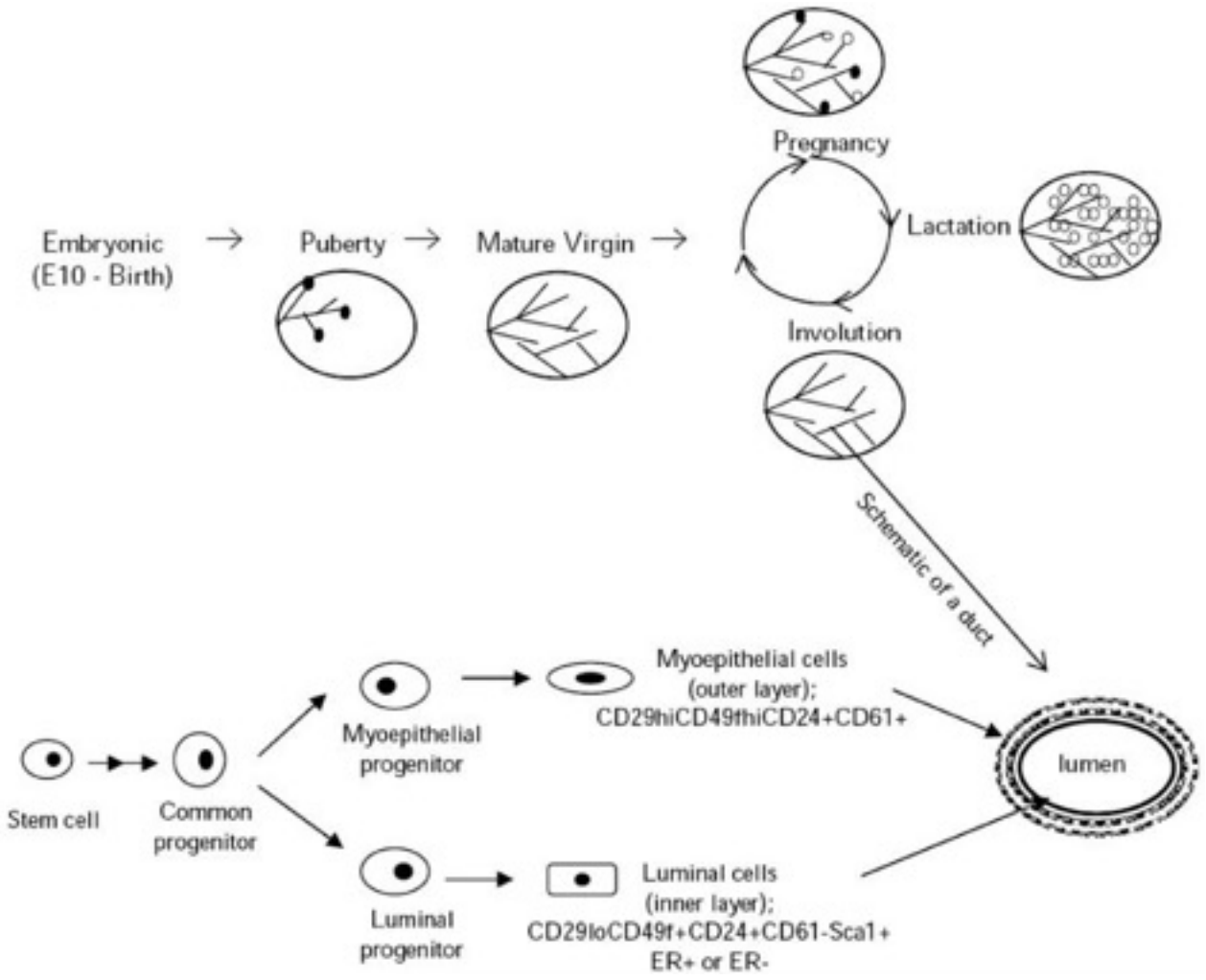
The number of stem cells must be maintained since they are the only cells capable of preserving the population and producing an offspring of differentiated cells

The Paneth cells produce factors such as epidermal growth factor (EGF), transforming growth factor a (TGF-alpha), and Wnt3, all essential for activation of the Wnt pathway and stem cell maintenance

Stress condition induce **symmetric division** of the SCs



STEM CELLS AND MAMMARY GLAND

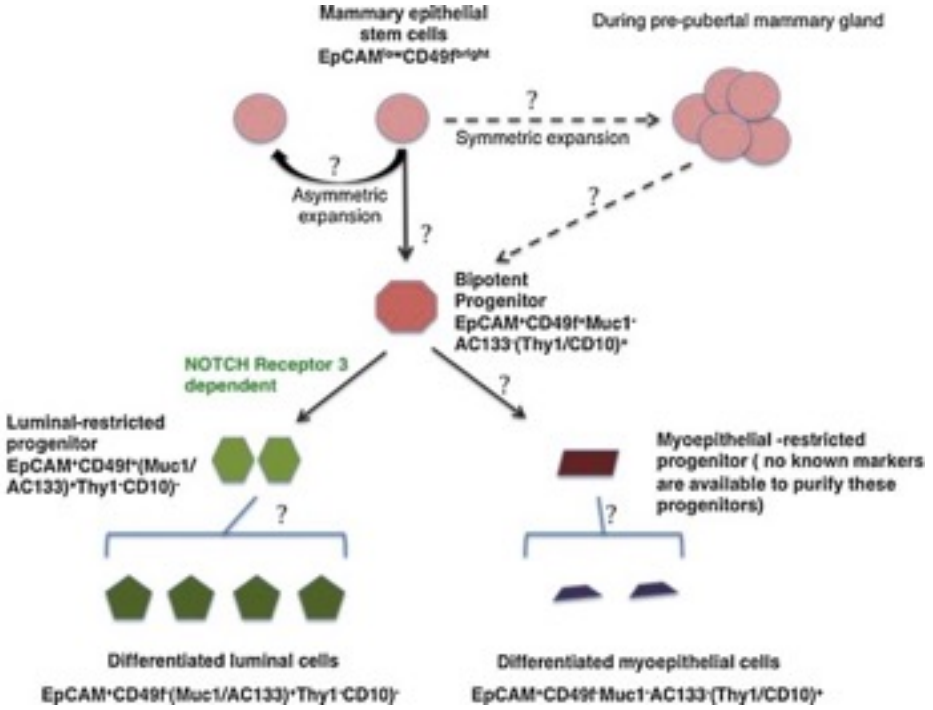


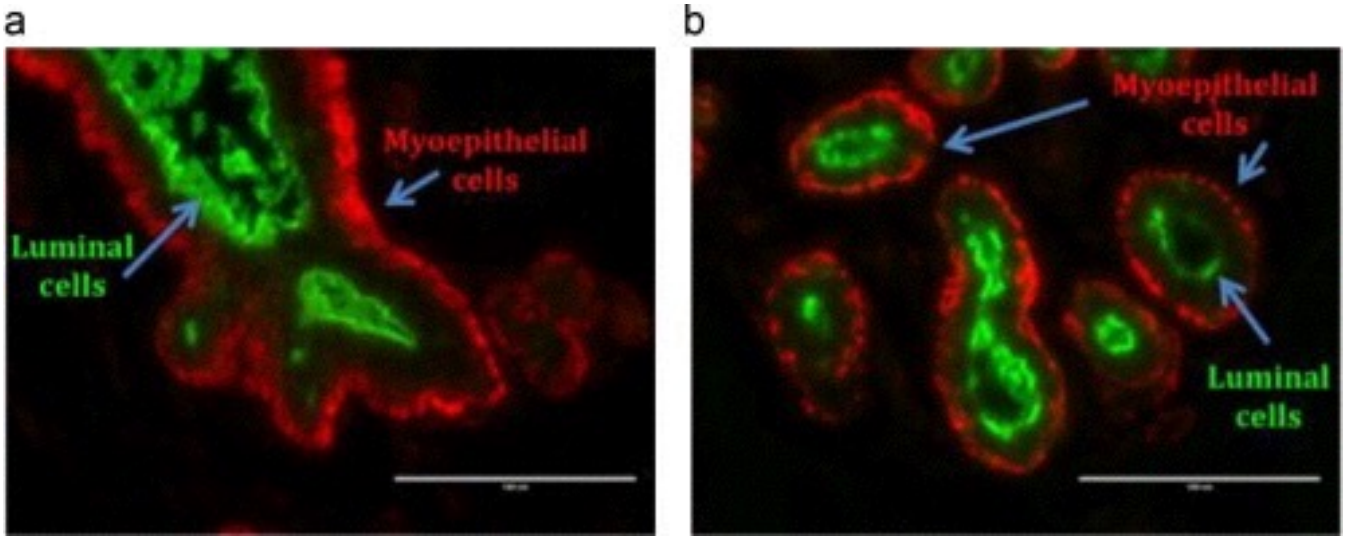
STEM CELLS AND MAMMARY GLAND

Cell surface markers CD44+/CD24- have been established as biomarkers for BCSCs

Upregulation of CD44 expression has been linked to breast tumor formation

The deregulation of Notch, Wnt/Frizzled/β-catenin, Hippo, and Hedgehog signaling pathways is believed to be involved in the formation of CSCs

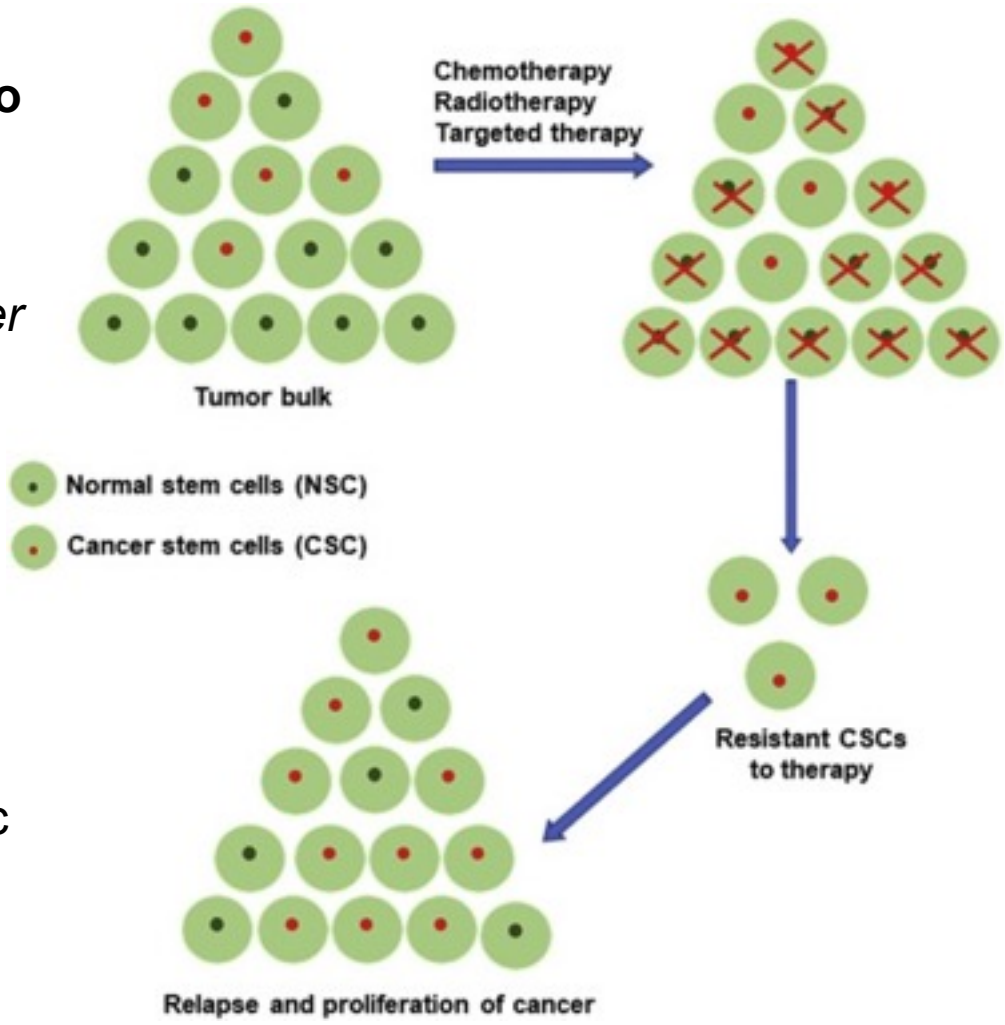


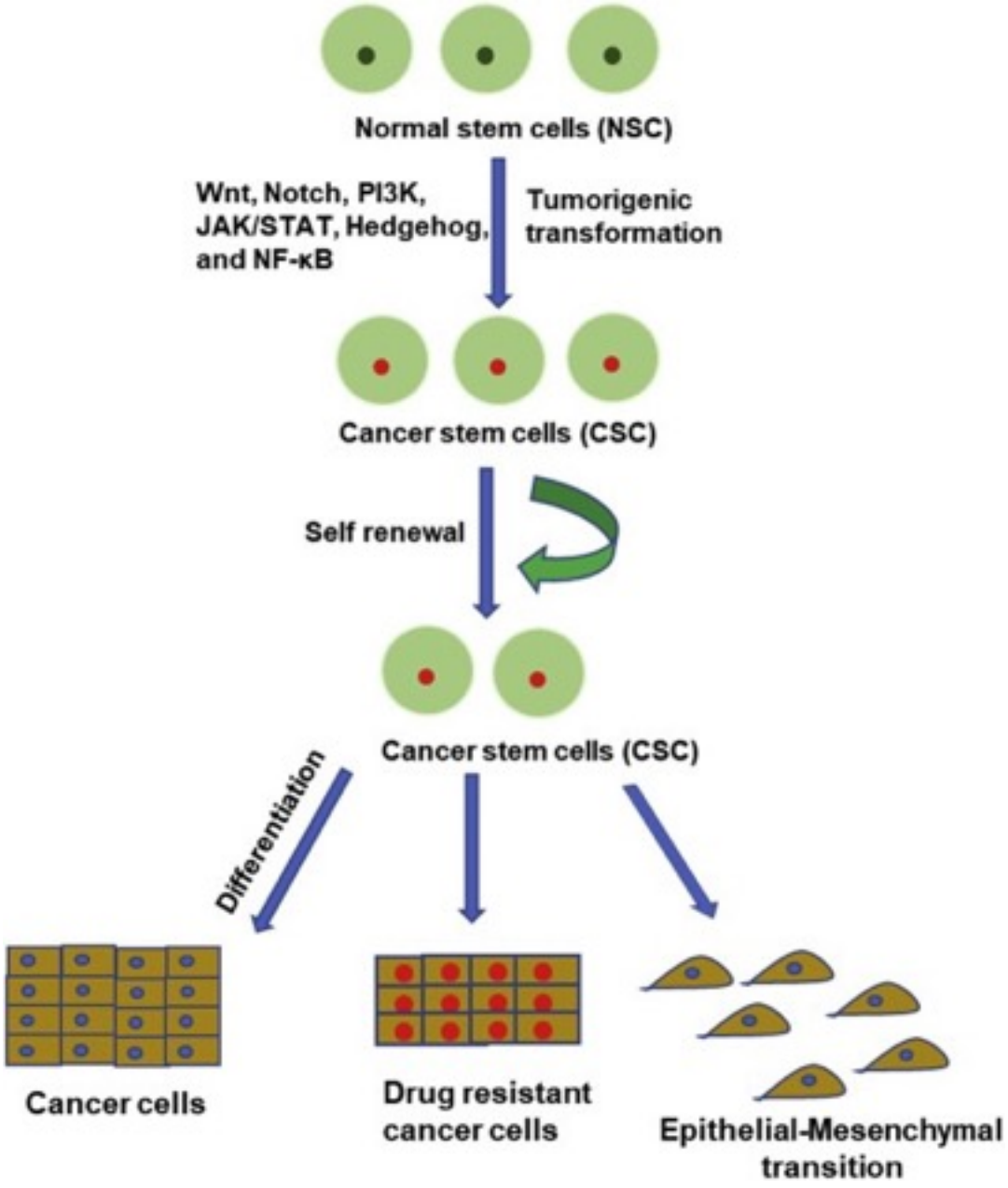


CSCs are generally resistant to chemotherapy and radiotherapy

A subset of remaining CSCs after therapy can survive and promote cancer relapse and resistance to therapies

CSCs role in drug resistance is crucial for establishing novel tumor diagnostic and therapeutic strategies.



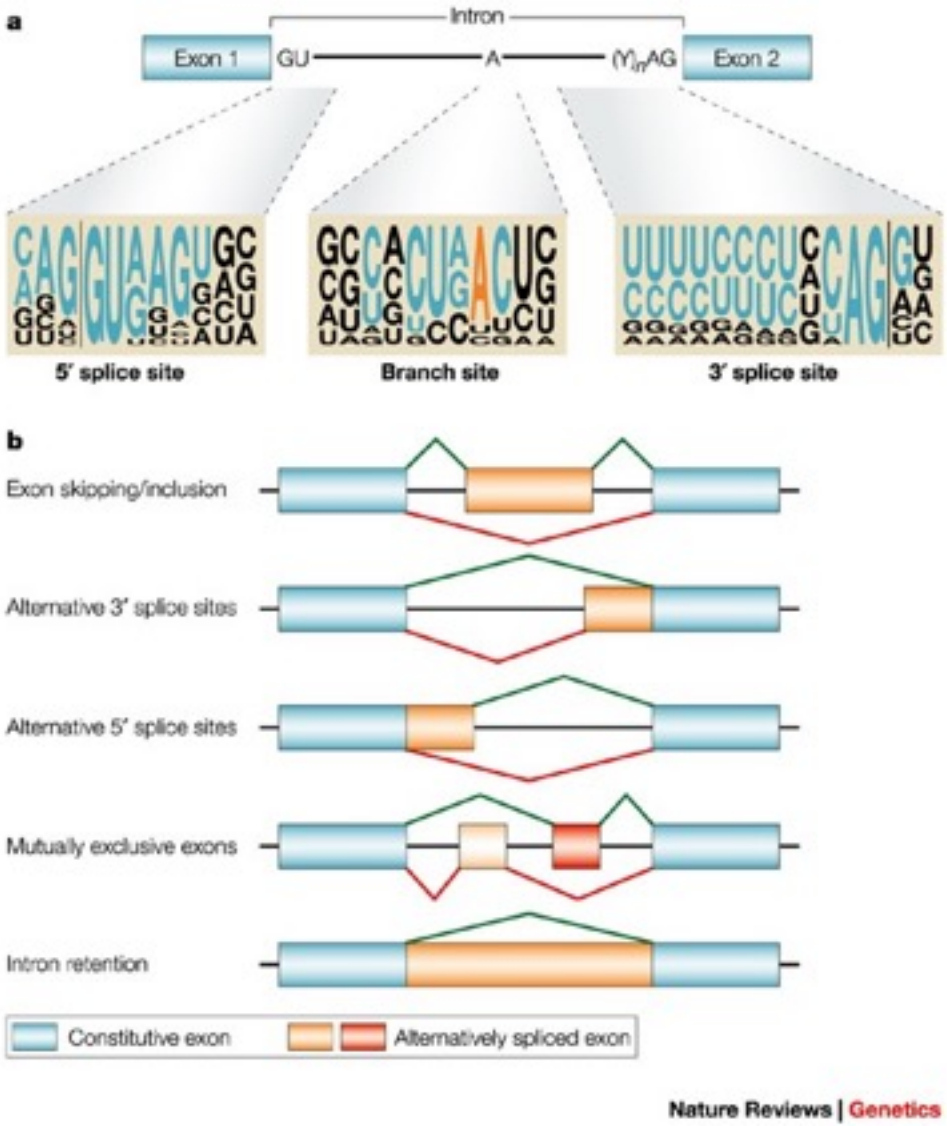

Normal stem cells (NSC)
Wnt, Notch, PI3K, JAK/STAT, Hedgehog, and NF-κB
Tumorigenic transformation
Cancer stem cells (CSC)
Self renewal
Cancer stem cells (CSC)
Differentiation
Cancer cells
Drug resistant cancer cells
Epithelial-Mesenchymal transition

ALTERNATIVE SPLICING

The production of several **isoforms** from the same transcriptional unit

Conserved motives near or flanking introns :

GU, AG, polypirimidine tract preceding 3'AG and A residues using as a branchpoint



Cartegni et al., 2002

Alternative spicing isoforms can modulate the phenotype of several genes

The percentage of mammalian genes affected by alternative splicing can vary between 22 and 74%

A subset of these isoforms are degraded as nonsense mediated mRNA decay (NMD)

Most gives rise to functional protein isoforms

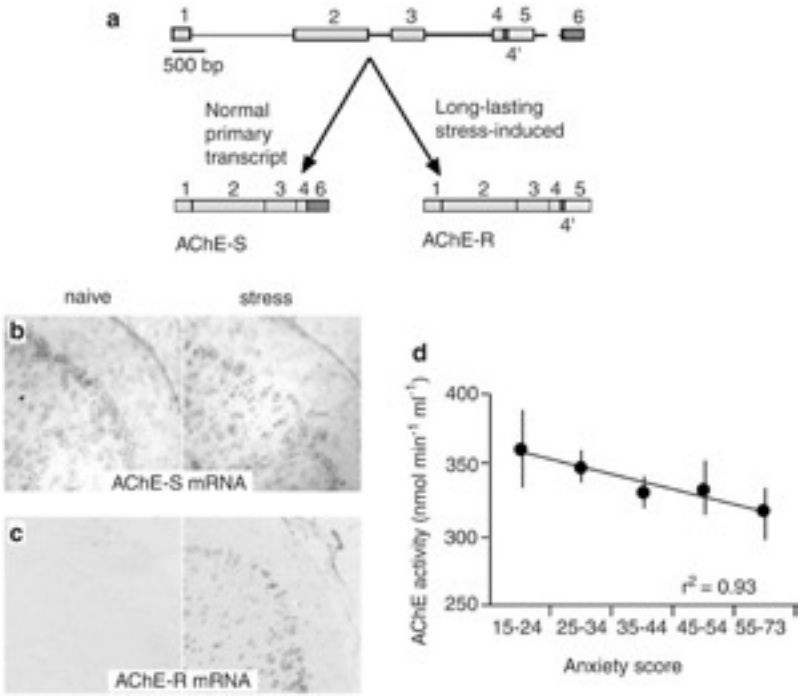
- Alternative transcripts are **less subjected to deleterious mutations** and are **hotspots for the evolution of proteins**
- Genomic variant can influence the **quality** and **quantity** of the alternative splicing

Alternative splicings can act as low penetrant alleles

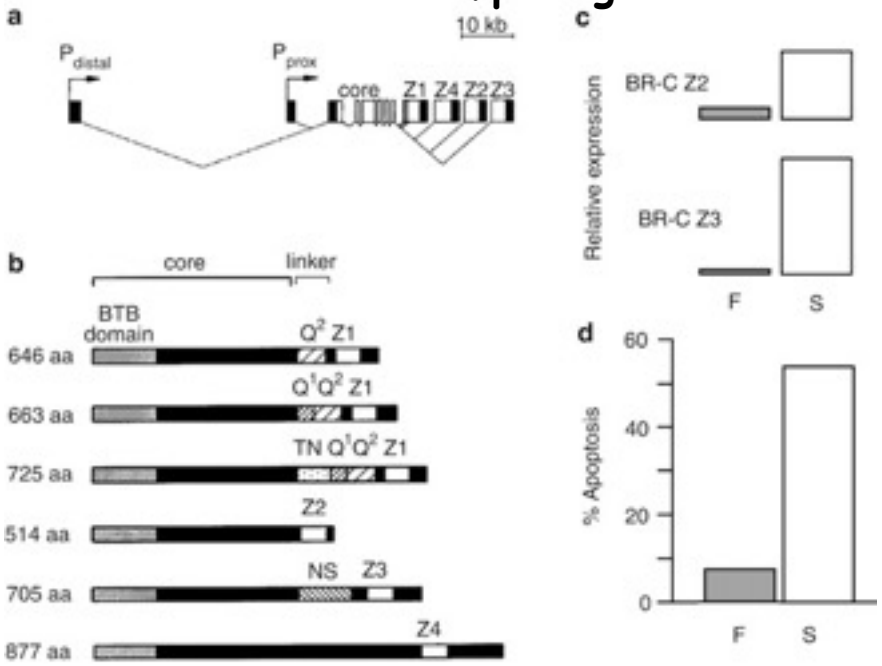
PHENOTYPE MODULATION

Quantitative variation of alternative splicing

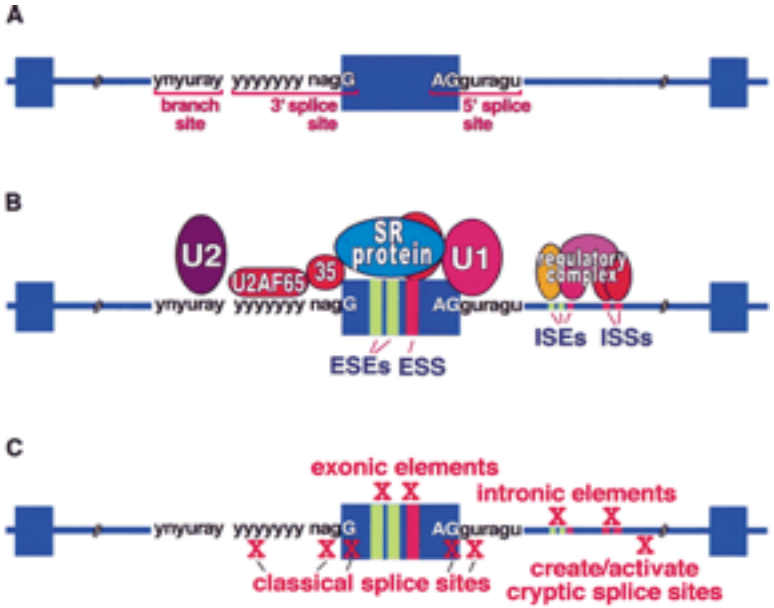
Neurologic replies to stress are associated with the differential splicing of the acetylcholinesterase gene (AChE)



The egg status and development are associated with alternative splicing of the Broad Complex gene



10-15% of the mutations involved in genetic disease are due to splicing alterations of pre-mRNAs



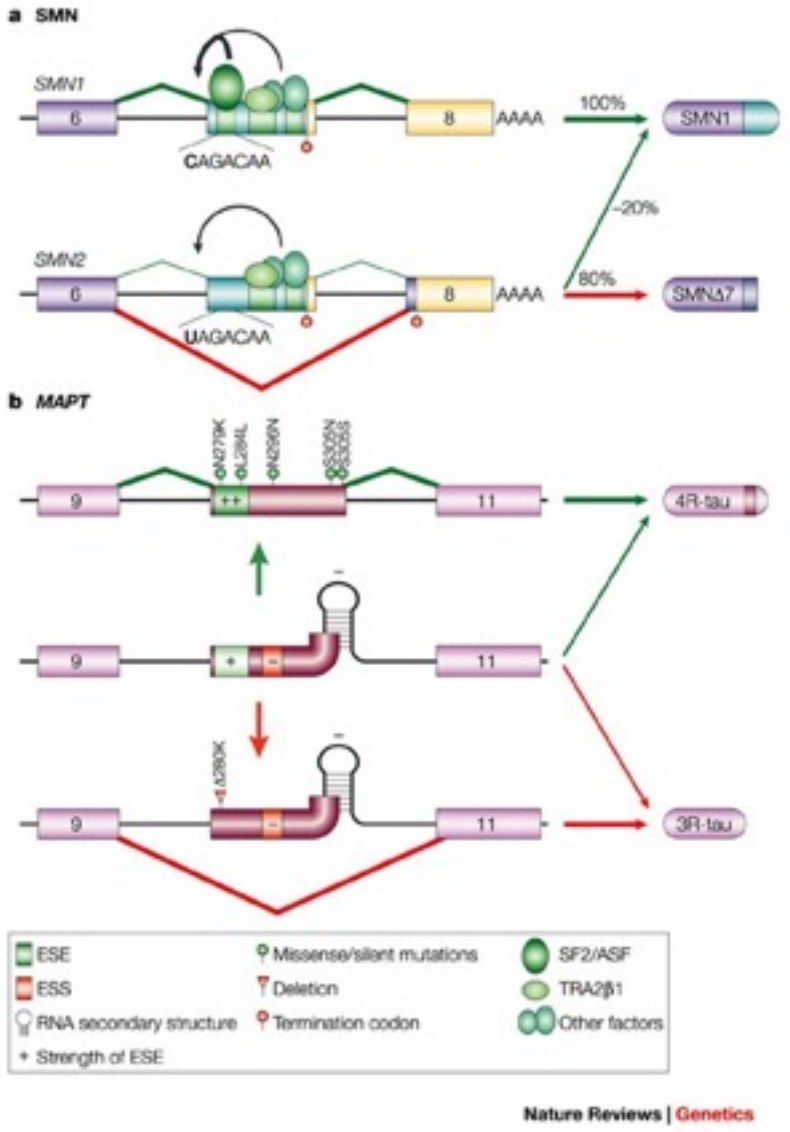
Aberrant splicings are due to variants playing in cis and in trans

- **In cis variants**: splicing sites, branchpoint points and supporting elements (es.ESE, ESS, etc)
- **In trans variants** : splicing machinary proteins involved in splicing regulation

Most of the pathologies show <u>in-cis variants</u>

- Splicing variants affect the conserved in-cis motives
- Missense or silent variants involve binding sites for the SR proteins regulating the splicing

Splicing alterations caused by exonic variants



a. SMN1 and SMN2 gene splicing are involved in <u>spinal muscular atrophy</u>

In SMN2 the silent transition C>T in position +6 of exon 7, inactivating an ESE sequence, causes an inefficient inclusion of this exon

b. MAPT splicing is involved in <u>dementia</u> and <u>parkinsonism</u> associated with chromosome 17

Exon 10 contains ESE and ESS elements. Sequences variants can alter the 4R-tau and 3R-tau ratio causing the disease

Cartegni et al., 2002

Genotype-phenotype correlation of splicing variants

Disease phenotype can correlate with **mRNA transcript level of splicing variants** and with the **ratio of alternative isoforms**

Disease	Gene	Mutation	Aberrant RNA	Level of aberrant RNA (%)	Phenotype tissue	Analyzed	Refs
MD	*ATP7A*	IVS6+1 G→A	Exon 6$^-$	100	Severe	Fibroblasts	[7]
		IVS6+5 G→A		100	Severe		
OHS		IVS6+6 T→A		95–98	Mild		
SMA	*SMN2*	IVS6+6 T→C	Exon 7$^-$	61	Type I	Lymphoblastoid cells	[17]
				59	Type II		
				47	Type III[a]		
CF	*CFTR*	3849+10kb C→T	+84 bp cryptic exon	50–99	Mild–severe[b]	Lung, pancreas, ileum, colon	[9,12]
CF	*CFTR*	IVS8–5T	Exon 9$^-$	63–94	Mild–severe[b]	Lung, epididymis	[10,12,13]
				76–94			
FD	*IKBKAP*	IVS20+6 T→C	Exon 20$^-$	100	Severe	Brain	[8]
				NA	Mild–severe[b]	NA	
Sandhoff	*HEXB*	P417L	Exon 11$^-$	20–40	Mild–severe[b]	Fibroblasts	[41]
PDH	*PDH E1α*	A175T	Exon 6$^-$	100	Severe	Fibroblasts	[42,43]
		G185G		50–80	Mild		
BMD	Dystrophin	R1314X	Exon 29$^-$	10[c]	Mild	Muscle	[44]
BMD	Dystrophin	E1211X	Exon 27$^-$	10–20	Mild	Muscle	[15]

Splicing alterations can be germline - associated with monogenic syndromes- and somatic –associated with poligenic diseases

Disease	Gene	Mutation	Splicing isoforms	Change	Analyzed tissue	Refs
FTDP-17	*Tau*	Δ280K IVS10+13/14/16 L284L, N296N N279K, S305N	Exon10+/−	↓ ↑ Brain	Brain	[20–22]
NF2	*NF2*	1737+3 A→T	Exon 15+/−	↓	Fibroblasts	[46]
Frasier	*WT1*	IVS9+4/5/6	KTS+/−	↓	Gonadal tissue	[47,48]
Wilms tumor	*WT1*	-	KTS+/−	↑	Tumor tissue	[49]
Wilms tumor	*WT1*	-	Exon5+/−	↓	Tumor tissue	[49]
Breast and ovarian cancers	*BRCA1*	G1694X	Exon18+/−	↓	Breast carcinoma cells	[16,50]
Breast cancer	*BRCA2*	-	Exon12+/−	↓	Breast carcinoma cells	[51]
Renal, lung and urothelial cancers	*CD44*	-	CD44v6-CD44v8+/−	↑	Tumor tissue	[52]
Gastric cancer	*CD44*	-	CD44v5, CD44v6+/−	↑	Serum	[24]
Papillary thyroid cancer	*CD44*	-	CD44v6-CD44v10+/−	↑	Papillary thyroid carcinomas	[53]
HNSCC, lung cancer	*FHIT*	-	Full length/ variable exon skipping	↓	HNSCC cells, lung cancer tissues	[54,55]
Invasive breast cancer	*MDM2*	-	Full length/ variable exon skipping	↓	Breast carcinoma	[56]
Giant cell tumors of bone	*MDM2*	-	Mdm2/mdm2-b	↓	Giant cell tumors of bone	[57]
Prostate cancer	*FGFR-2*	-	IIIb/IIIc	↑	Prostate cancer cells	[58]
Melanoma	*Bin1*	-	Exon12A+/−	↑	Melanoma cells	[59]
Prostate cancer, lymphoma, gastric carcinoma	*Bcl-2*	-	Bcl-2α/β	↑	Prostate cancer cells, follicular lymphomas, gastric carcinoma	[23]
Lymphoma, breast cancer	*Bcl-x*	-	Bcl-xL/S	↑	Lymphoma cells, breast carcinoma	[23]
Oral and oropharyngeal cancers	*Bax*	-	Bax-α/ω	↓	Oral and oropharyngeal carcinomas	[23]

APC gene altered transcript level in AFAP patients negative for APC truncating mutations

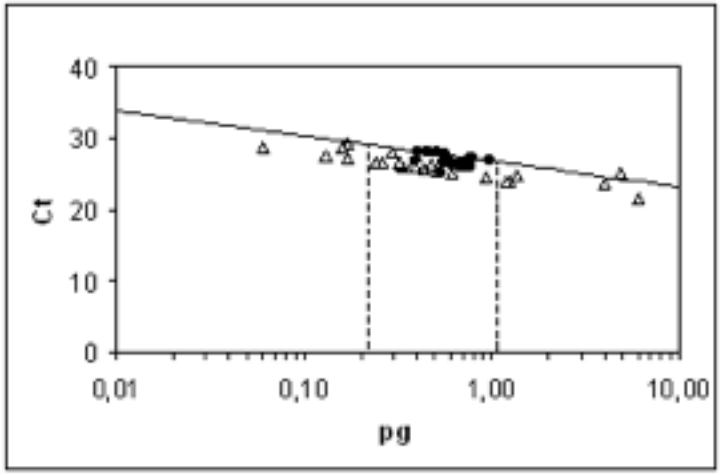
CASES : **26 AFAP patients** without APC truncating mutations

9 patients < 15 adenomas + 11 patients > 15-70 adenomas + 6 patients <70-100 adenomas

CONTROLS : **20 healthy subjects without a family history of polyposis or CRC**

1. APC mRNA level in AFAP patients vs healthy controls

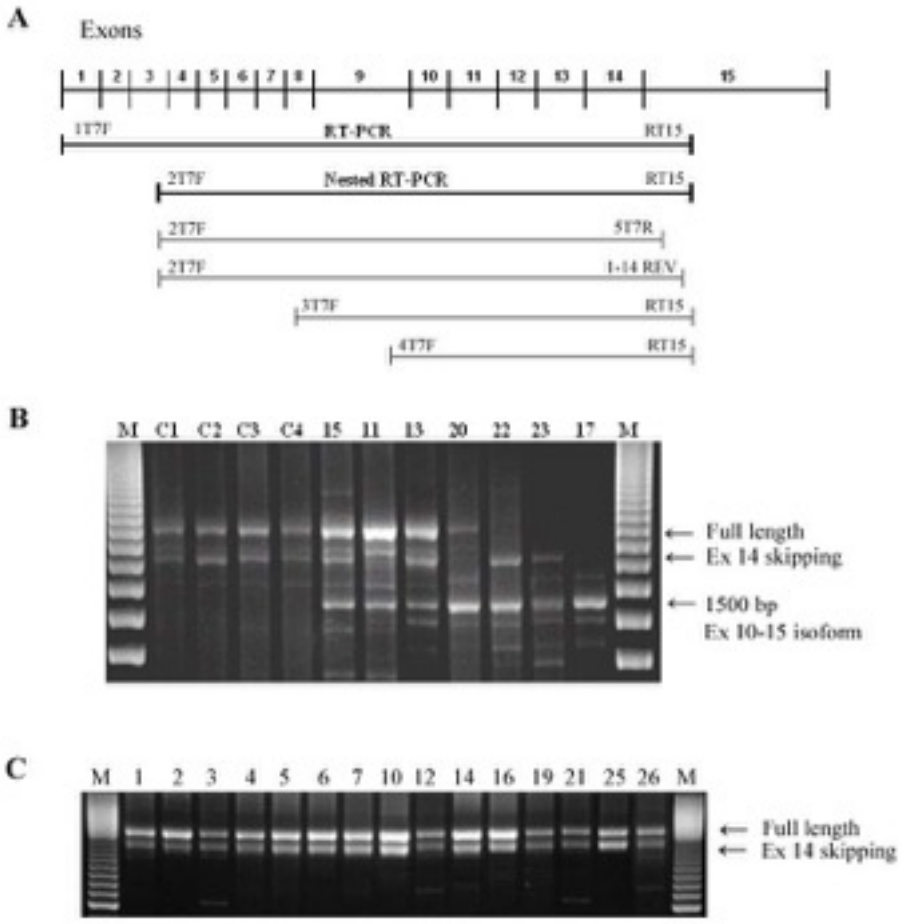
We checked APC gene transcript level by Real Time RT-PCR (Taq-Man method)



Patients	mRNA pg
8	0.06±0
9	0,13±0,01
11	0,17±0,02
13	1,24 ±0,21
15	1,20±0,4
17	0,17±0,03
18	4,86±1,67
20	1,35±0,26
22	4,01±0,7
23	0,16±0,03
24	6,04±0,78

11 AFAP patients showed mRNA APC < 0.22 pg or > 1.11 pg

2. APC alternative transcripts by nested RT-PCR



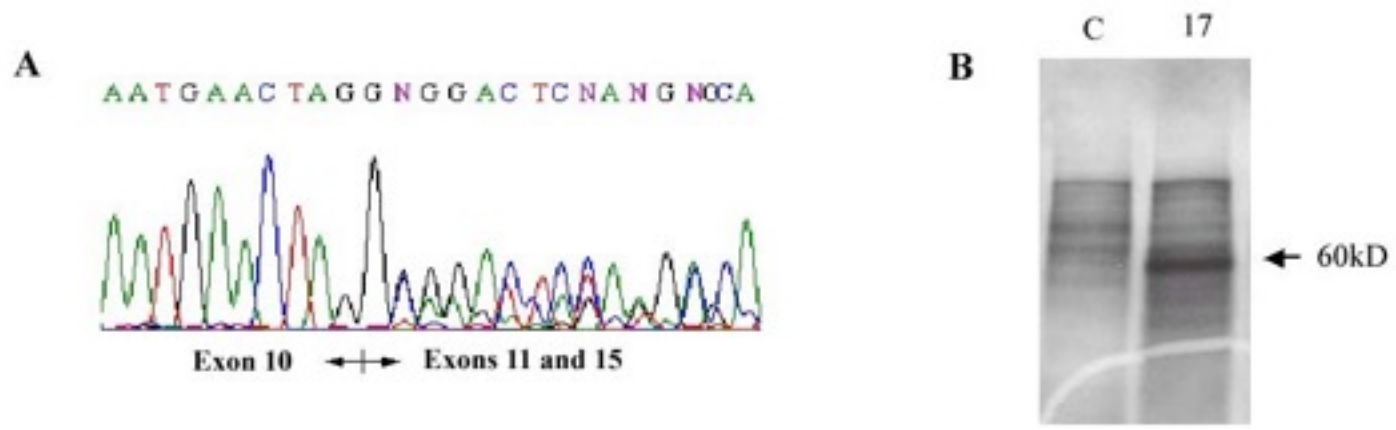
In 4 patients (20, 22, 23, 17) the high expression of a new transcript was associated with a decreased expression of the full-length transcript

Healthy controls

3. Sequencing of APC alternative transcripts

Direct sequencing of RT-PCR products evidenced an alternative splicing joining exons 10 and 15, reported in colorectal cell lines (Sulekova et al. 1995).

This splicing caused the loss of the reading frame with the insertion of a stop codon at the beginning of exon 15



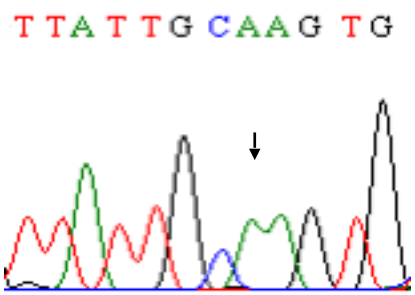
By using specific primers mapping on exon 10 and 15, we evidenced the presence of this splicing also in the control populations

4. The stability of this transcript was checked by Protein Truncation Test

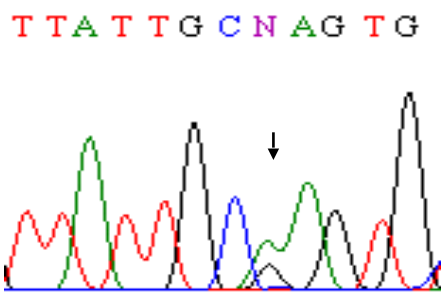
PTT was performed on the nested RT-PCR fragment joining exons 10 and 15.

It showed the presence of a truncating protein product

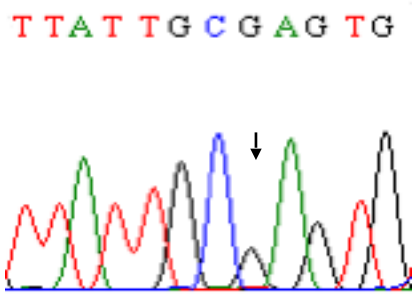
5. A silent variant was identified in a ESE sequence of exon 13 (1635 A>G)



1635 A/A



1635 A/G



1635 G/G

This A>G transition is able to destroy an ESE motif and decreases the affinity of this region for the SRp40 protein which is involved in the splicing mechanism.

SR	Variant	Protein	Cut-off significity	Wild-type significity	Variant significity
	1635 A→G	SRp40	2,67	2,83	0,28

Three AFAP patients showing this splicing were heterozygous A/G and one was homozygous G/G

Web softwares for the identification of nucleotide substitutions affecting splicing (in silico analysis)

ESE finder, RescueESE* and *PESX

In-silico splicing predictions do not always correlate with in-vivo data

InSIGHT database reported 382 mutations for hMLH1 and hMSH2

A lot of these alterations were nonsense or frameshift pathogenetic mutations

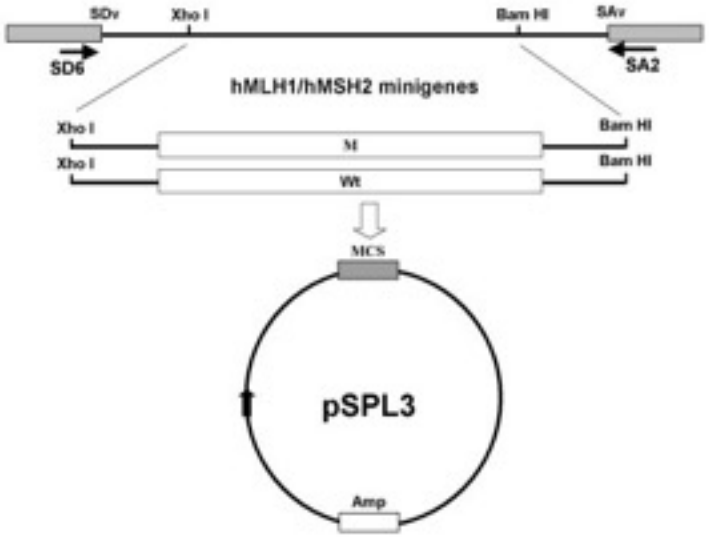
Most of the **missense** mutations were located in **ESE** (Gorlov et al., 2003)

Lastella et al., 2006

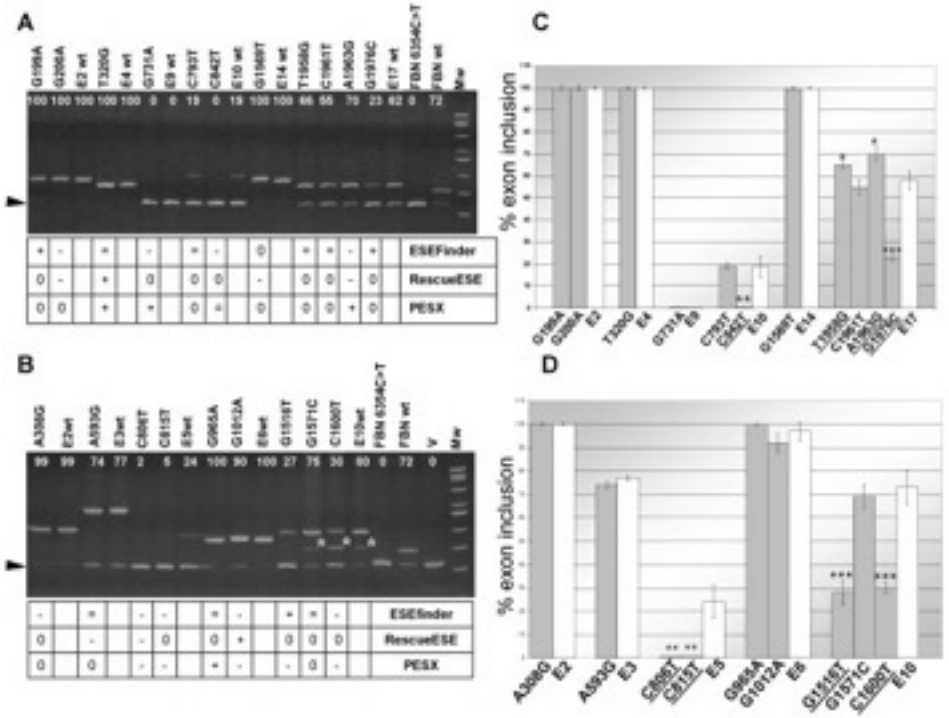
To assess the correlations of in silico splicing predictions with in vitro results

- 99 hMLH1 and hMSH2 missense mutations reported in database were analyzed with 6 different algorithm (ESE finder, RescueESE and PESX etc)
- 20 of these alterations were also tested by in vitro analysis

Reporter constructs were assessed to carry out splicing assays



Cos-7 cells were transfected



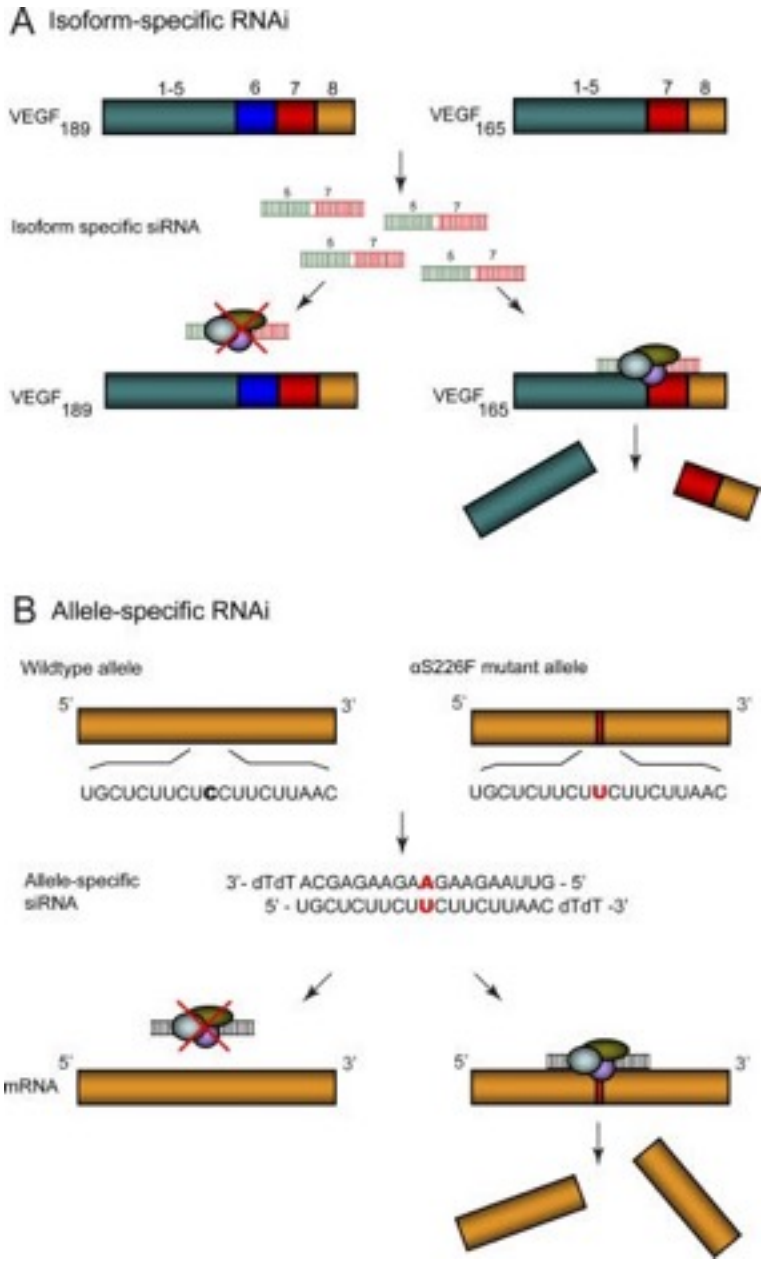
- **8 mutations were associated with splicing alterations**
- **6 caused a 50% decrease of their exon inclusion rate**

RNA interference : a potential therapy for disease associated isoforms

- Selected siRNAs for mRNAs associated specifically with the disease
- Exon-exon junction siRNAs are the more difficult to project

2 different approaches:

- Knockdown using RNAi specific for splicing isoforms
- Knockdown using RNAi for allele variants

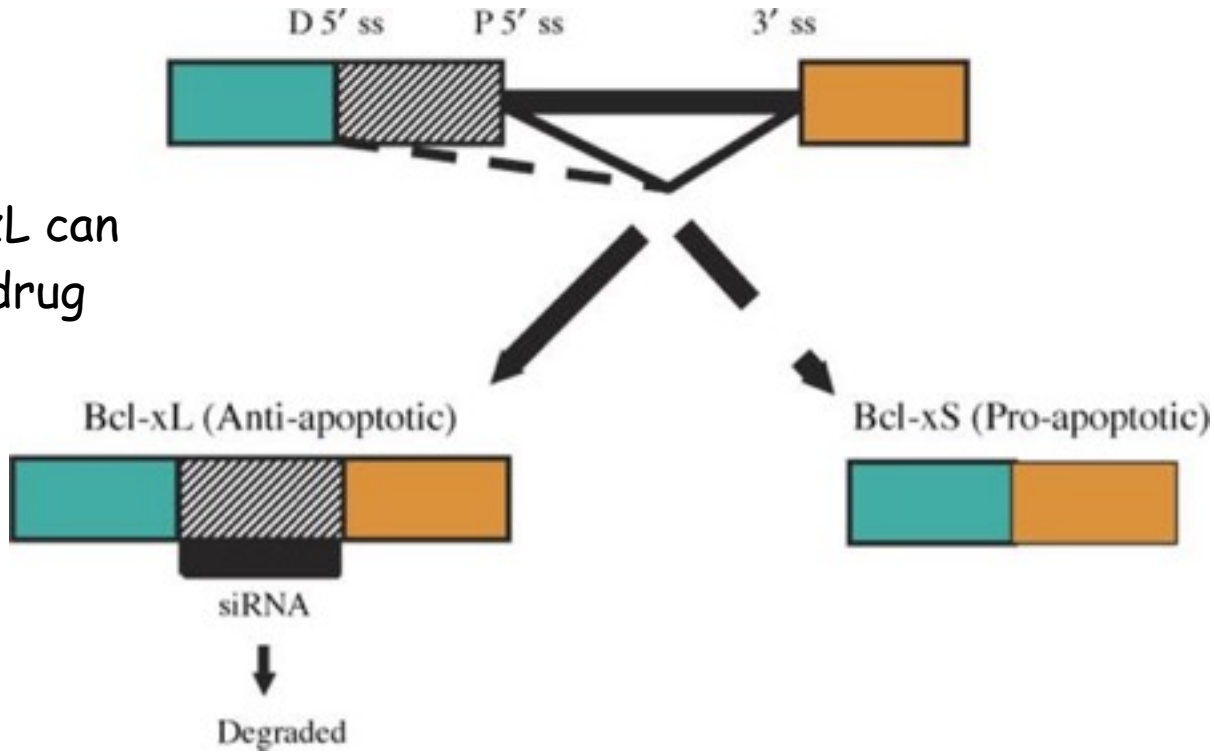


Downregulation of splicing isoform: Bcl-xL

Bcl-X, a member of Bcl-2 family,is a apoptotic regulator

2 isoforms: Bcl-xL, anti apoptotic and Bcl-xS, pro-apoptotic

RNAi knockdown of Bcl-xL can be used as anti cancer drug



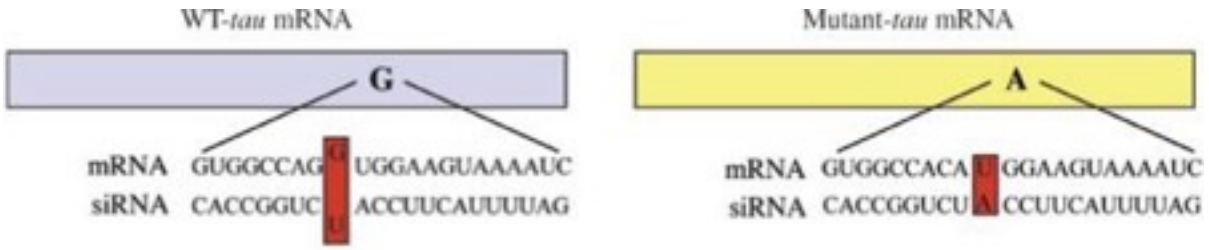
Downregulation of allele variant:

tau gene

Some tau mutations cause aberrant splicings,

others led to the expression of aberrant proteins

tau V337M mutation, on exon 12 is associated with frontotemporal dementia (FTD)



A specific siRNA can inactivate the mutant mRNA allele without affecting the expression of the normal allele