

DIAGNOSTICA MOLECOLARE CLASSICA E NGS NELLE LAM

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MOLECULAR DIAGNOSIS: main molecular techniques conventionally used

PCR

Real Time-PCR (RT-PCR)

Sanger Sequencing

DHPLC

SNP array

AML: molecular diagnosis

t(8;21)
Inv16
PML/RARA

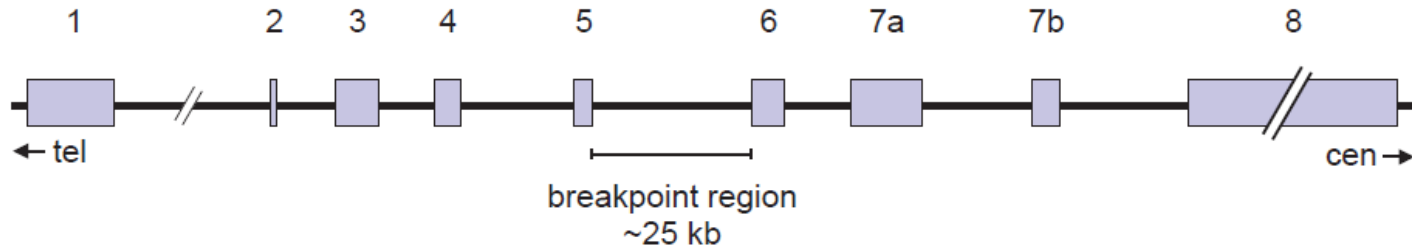
**Conferma
alterazioni
citogenetiche**

FLT3
NPM1
WT1
CEBPA
RUNX1
IDH1/2
TET2

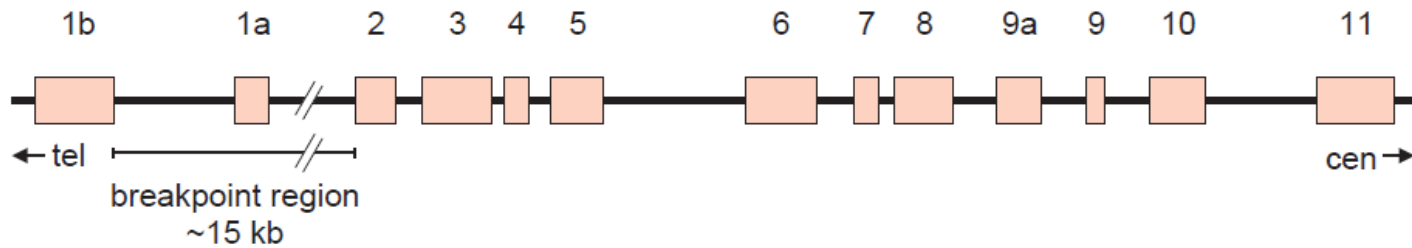
**Altre
alterazioni
molecolari**

t(8;21) AML1-ETO

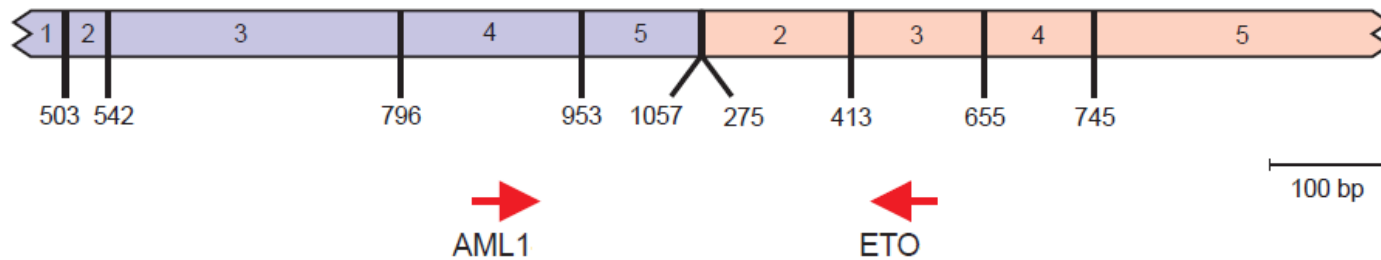
AML1 (21q22)



ETO (8q22)



b



Molecular variants and their prognostic value

- Mutazioni

FLT3

IDH1, IDH2

RUNX1

TP53

- Espressione

MN1

BAALC

WT1

ABCG2

**Prognosi
sfavorevole**

NRAS

KRAS

TET2

ASXL1

CBL

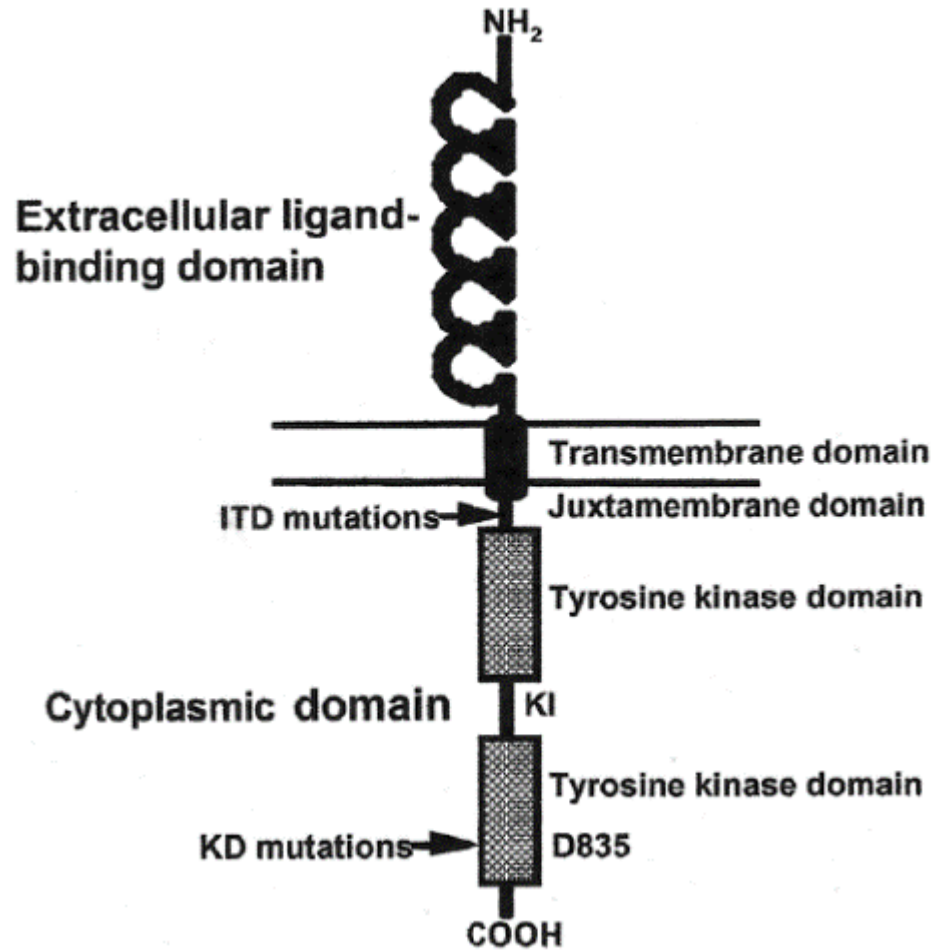
**Significato
prognostico
da definire**

NPM1

CEBPA α

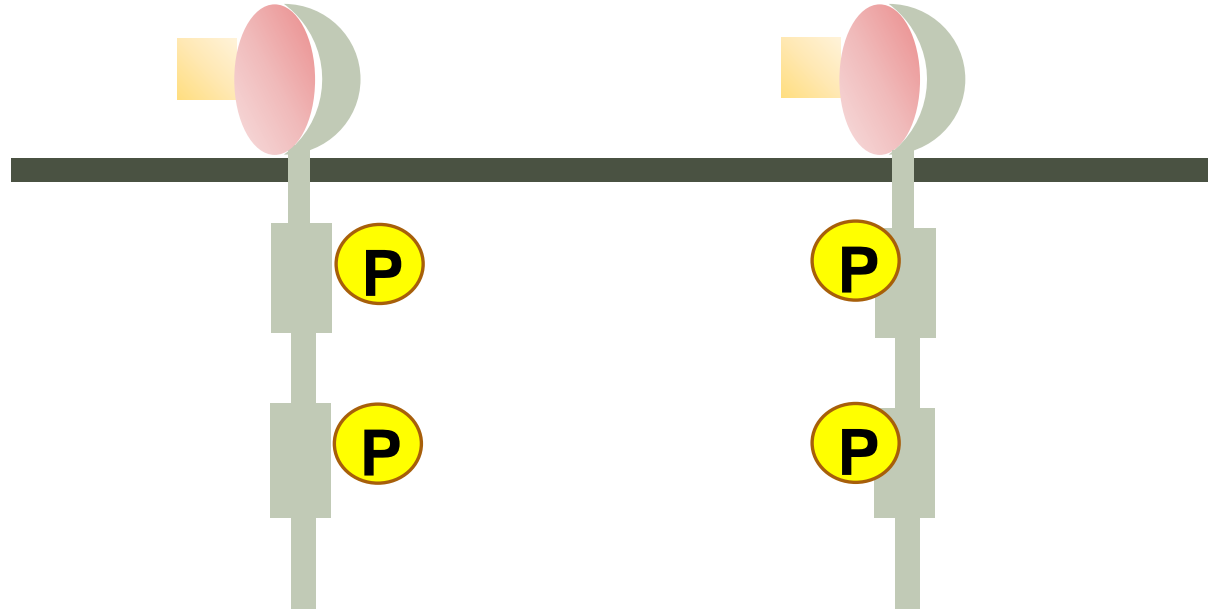
**Prognosi
favorevole**

FLT3



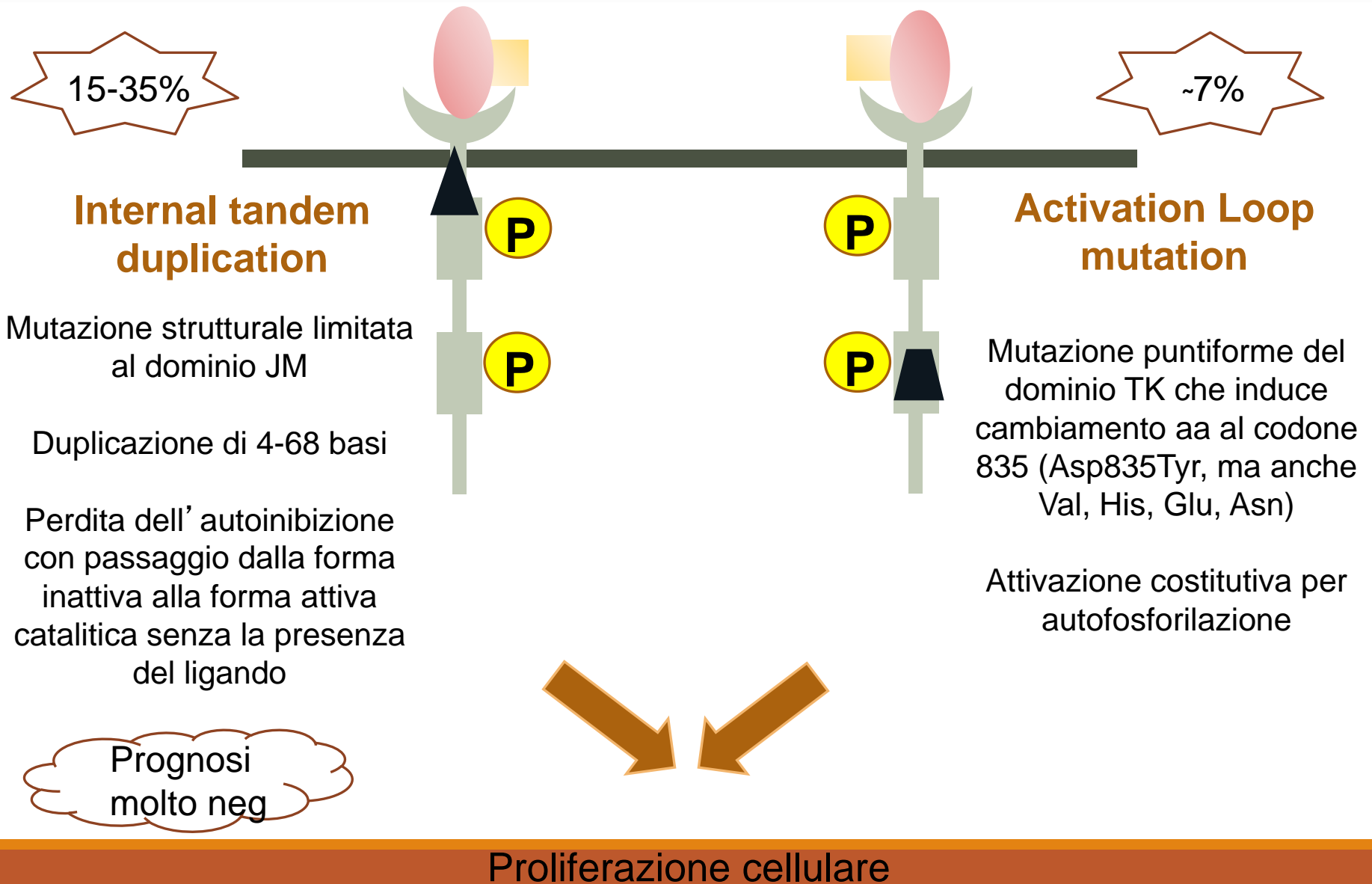
FLT3

Primariamente espresso sulle cellule empoietiche immature, ed è essenziale per la normale funzione delle cellule staminali

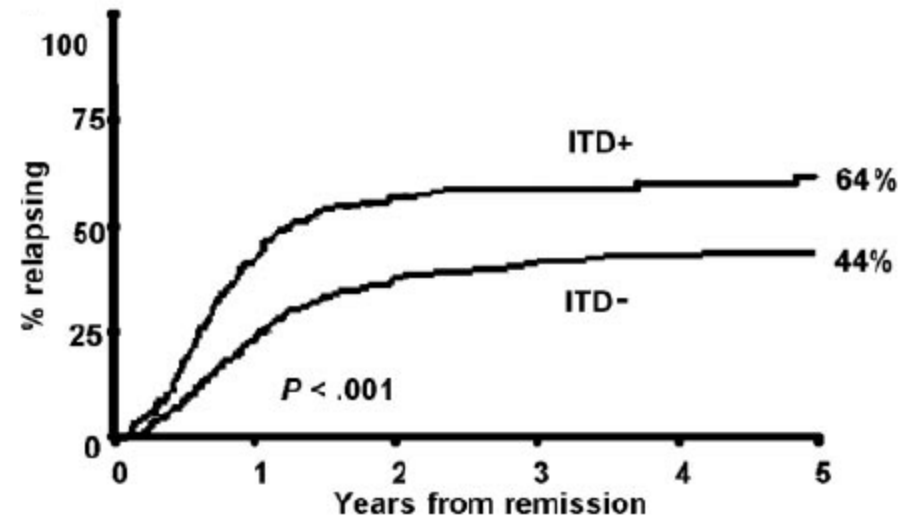
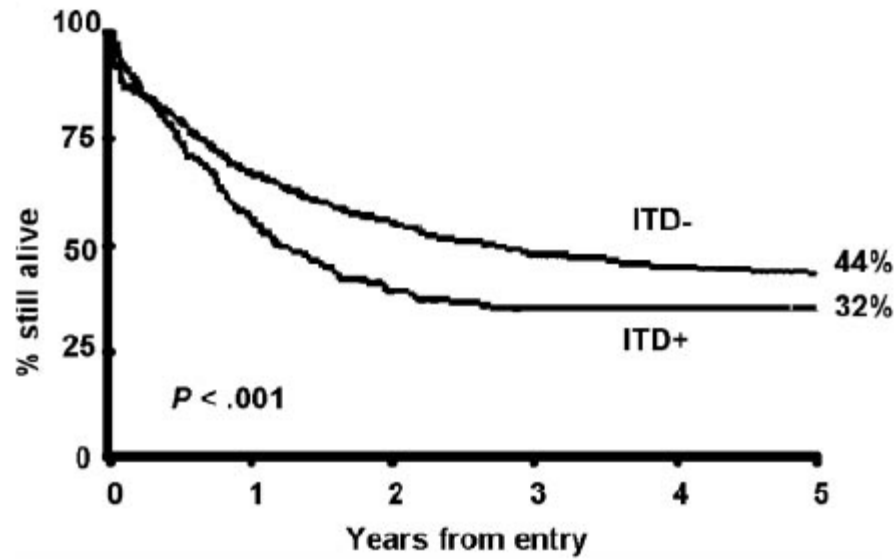


Regolazione della differenziazione, sopravvivenza, proliferazione e apoptosi

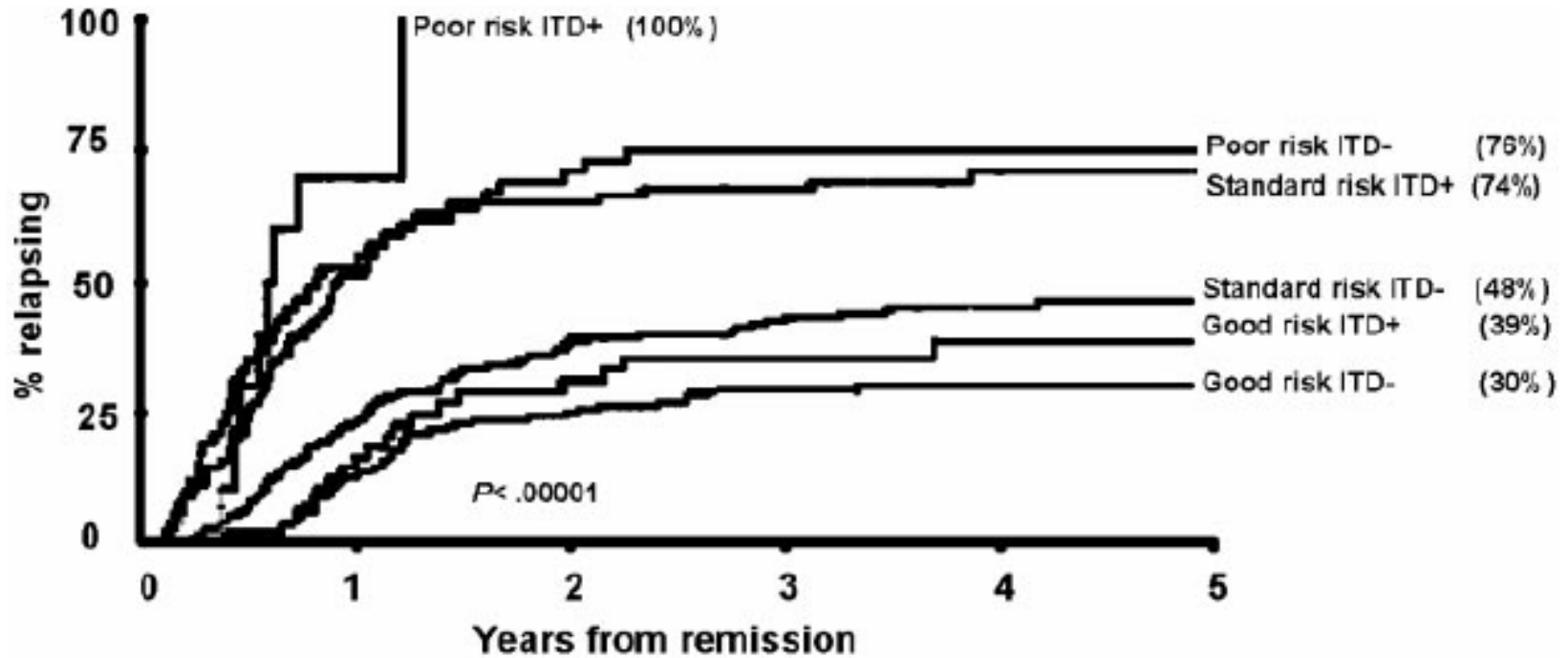
FLT3



FLT3

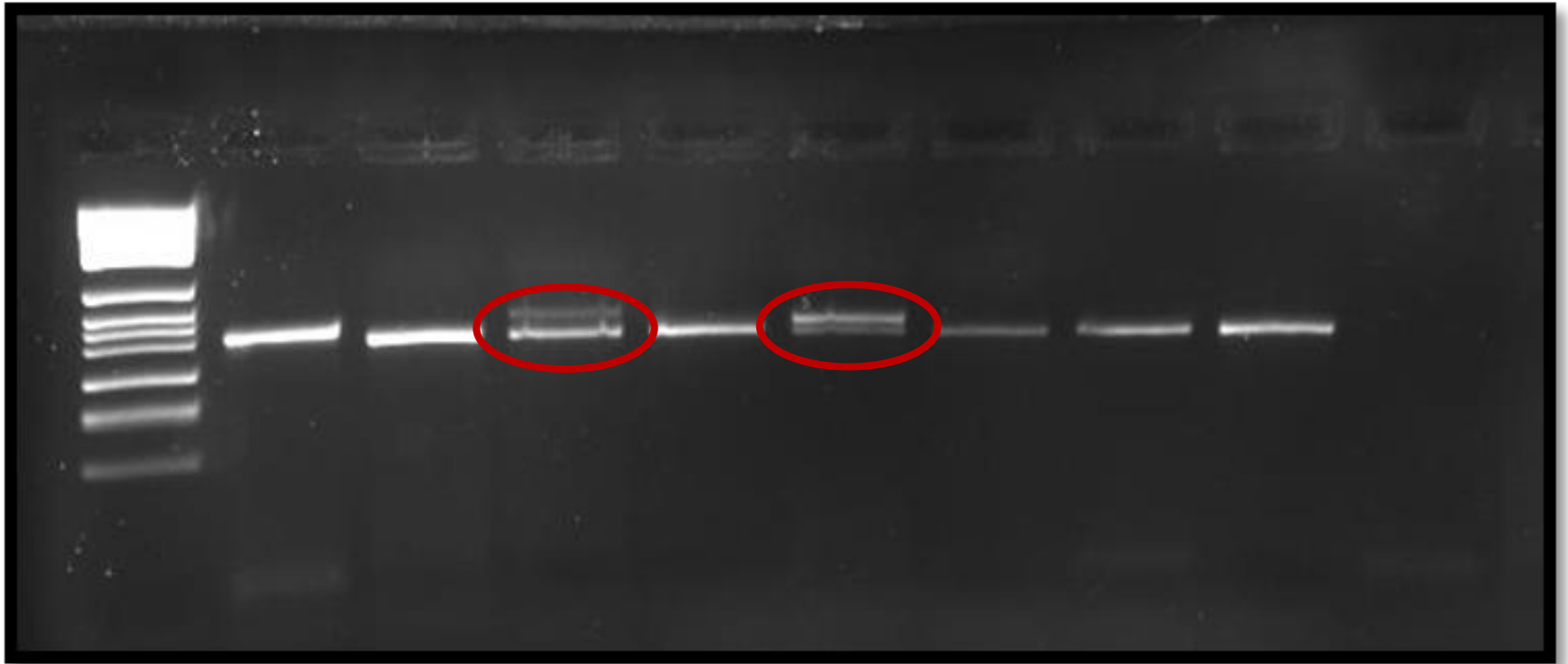


FLT3



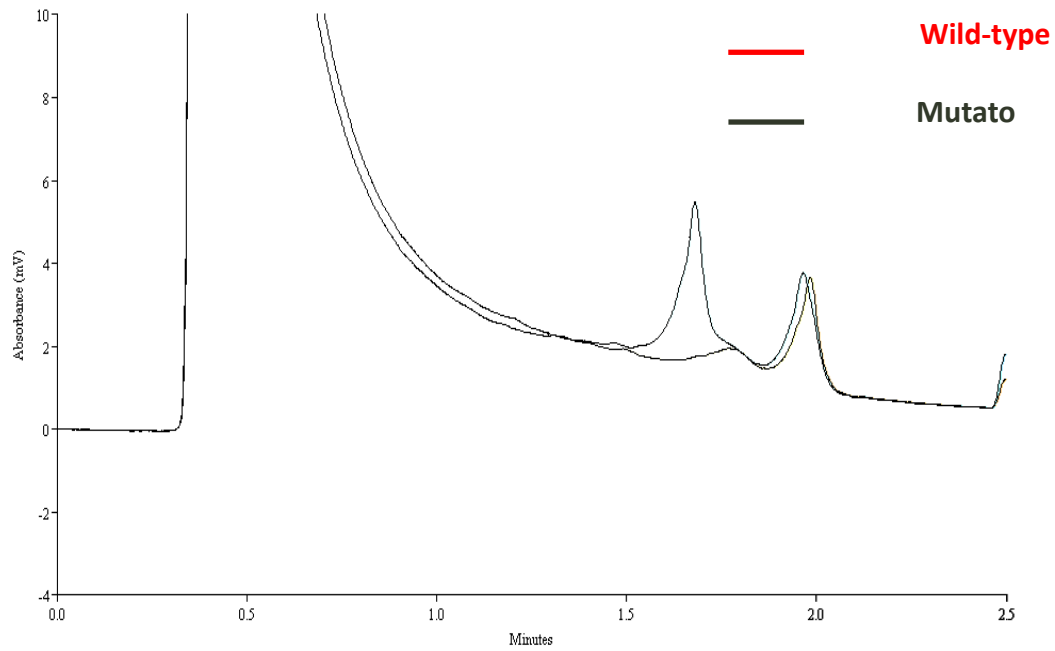
FLT3

1. PCR qualitativa



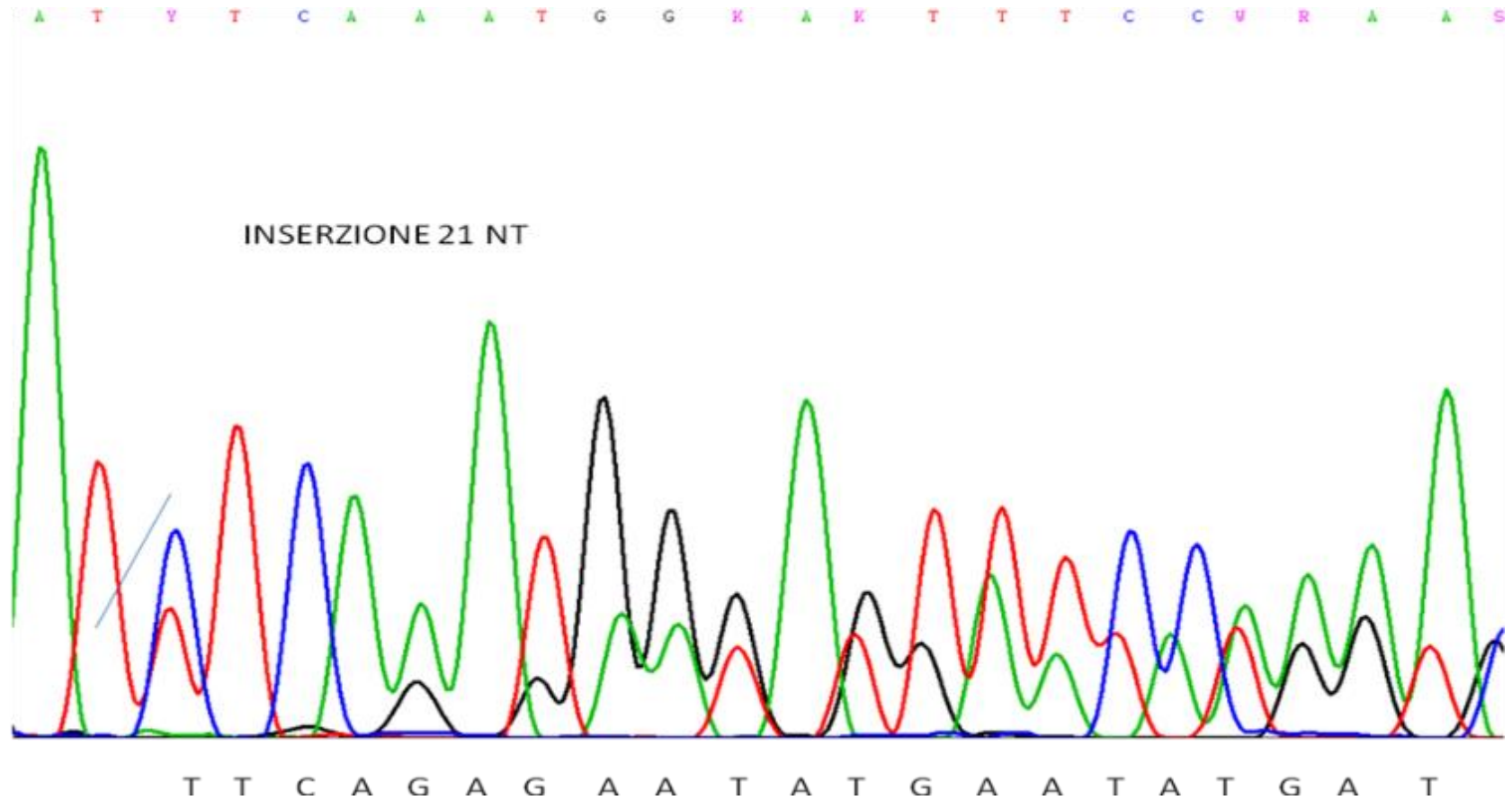
FLT3

2. DHPLC



FLT3

3. Sequenziamento convenzionale



NPM1

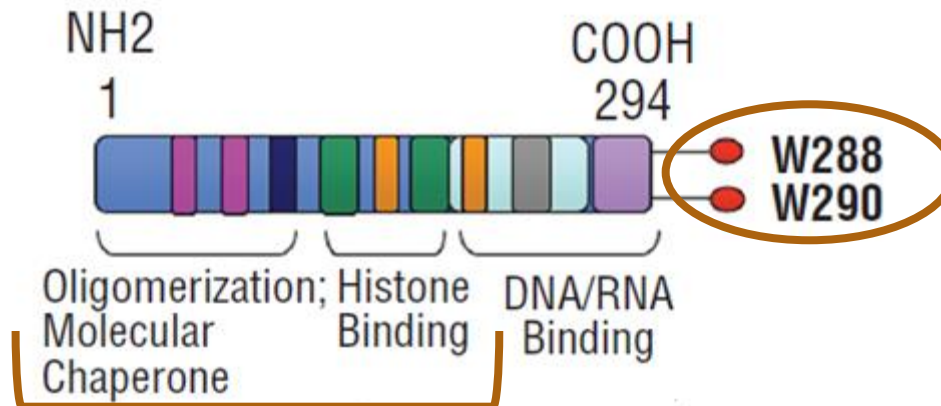
Fosfoproteina con attività di oncosoppressore, ubiquitariamente espressa nei nucleoli

Svolge attività di chaperone

previene l'aggregazione di proteine nel nucleolo

favorisce l'assemblaggio di istoni e nucleosoma

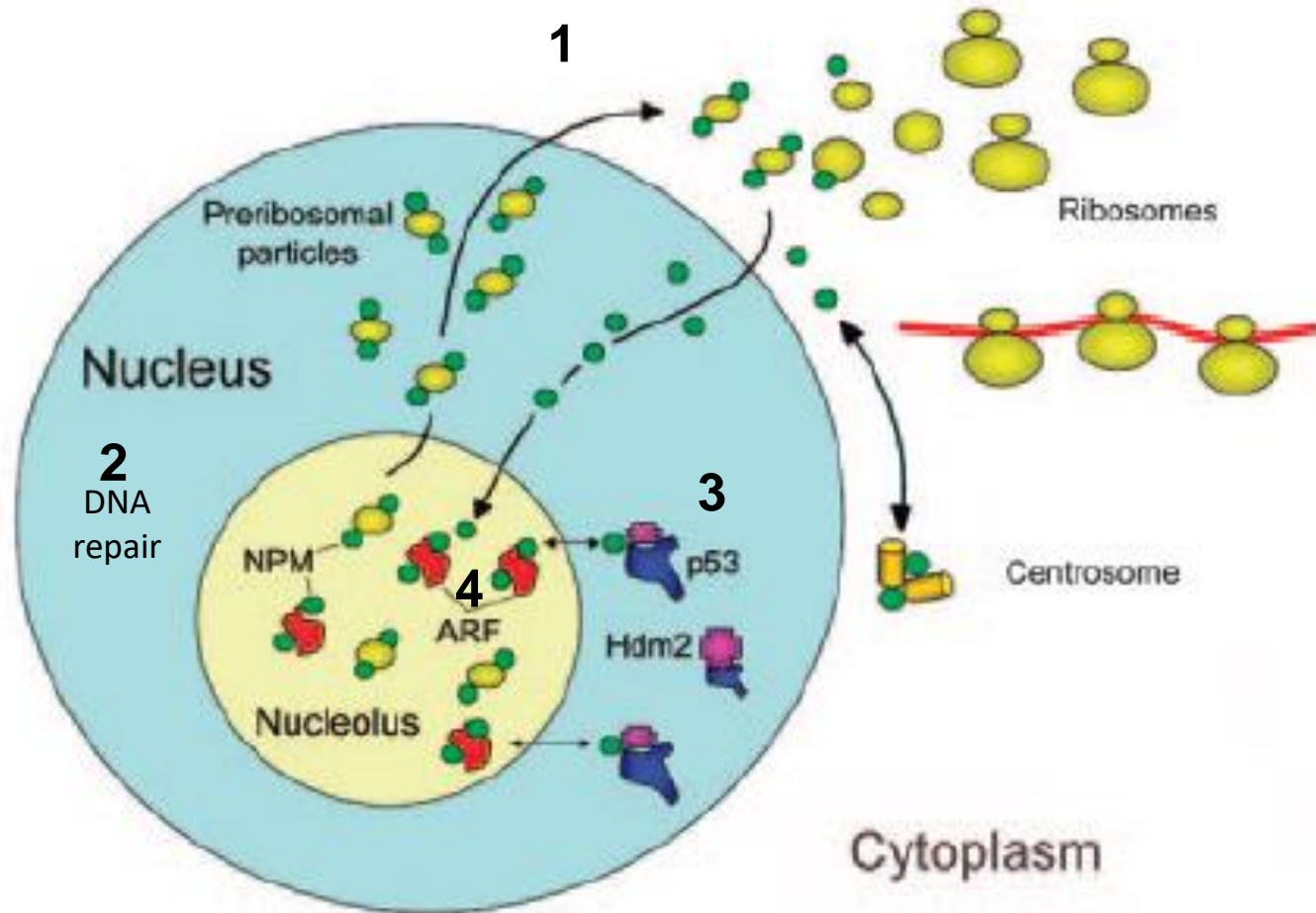
aumenta l'attività trascrizionale acetilazione-dipendente



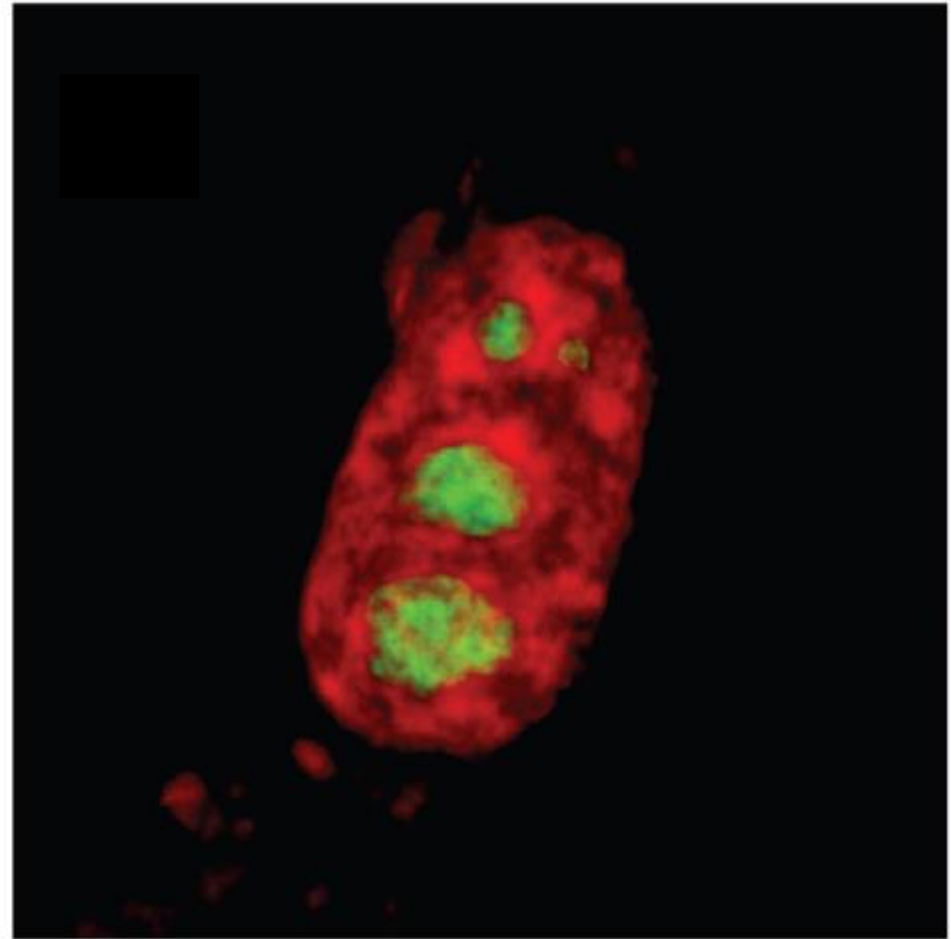
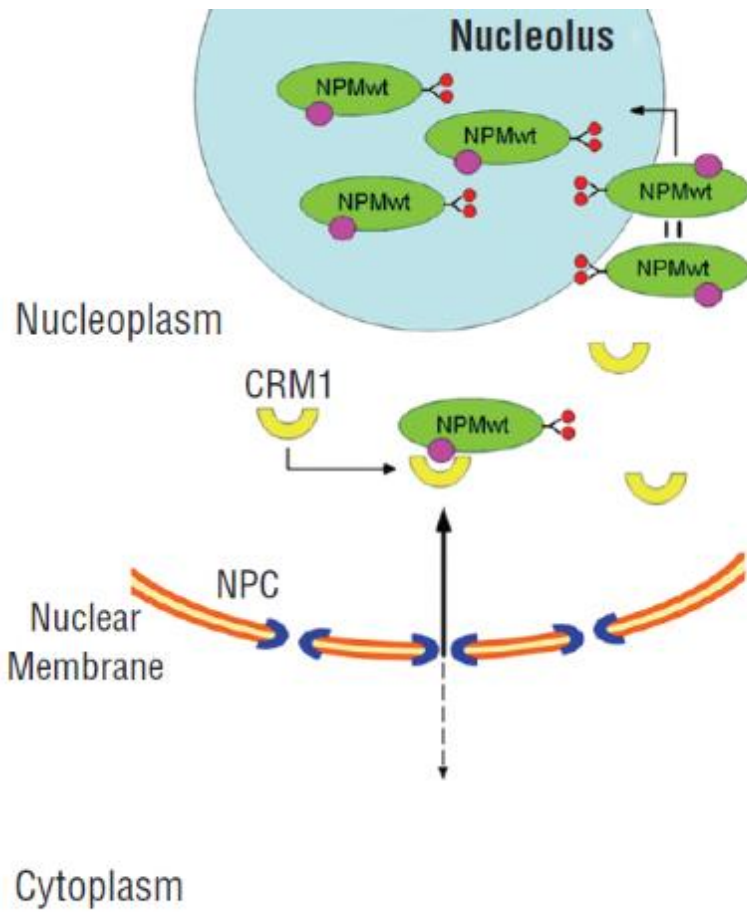
Due triptofani
cruciali per il
legame con il
nucleo

Domini necessari per la
funzione di chaperone

NPM1



NPM1

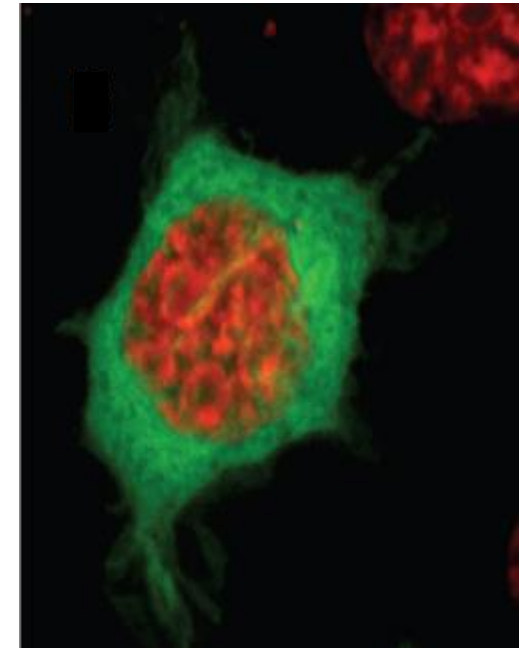
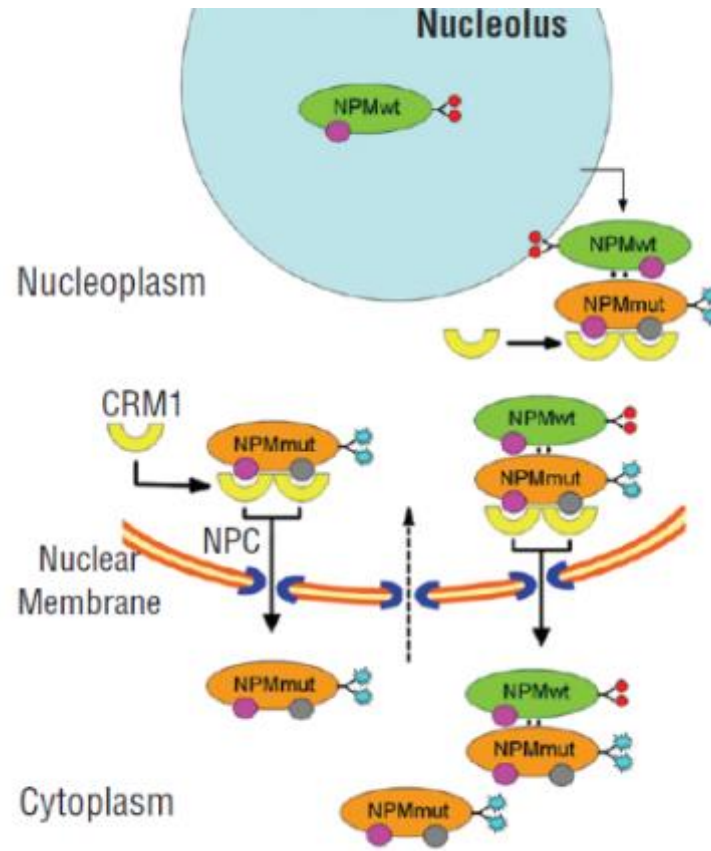


NPM1

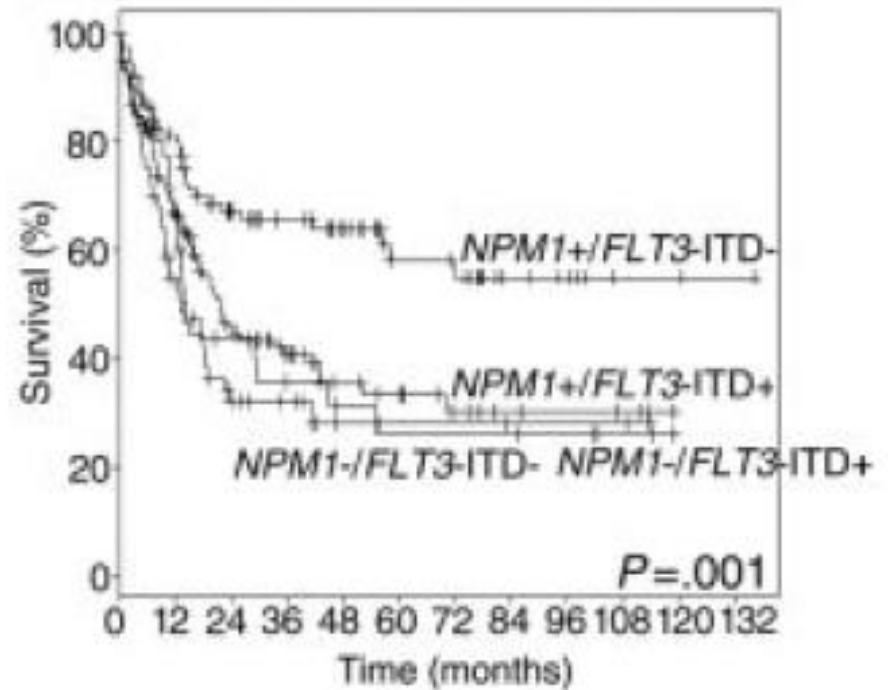
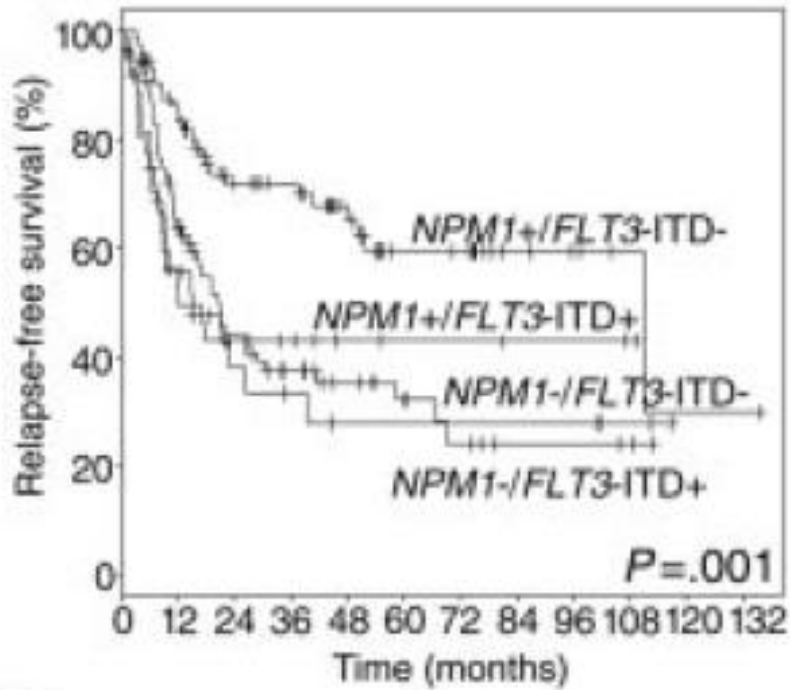
Descritte >40 mutazioni (solitamente nell' ex 12, ma possono interessare anche il 9 o l' 11). La più frequente (70-80% delle mutazioni nelle AML degli adulti) è una duplicazione del tetranucleotide TCTG in posizione 956-959



Tutte creano un mutante con un nuclear export signal nella porzione C-terminale



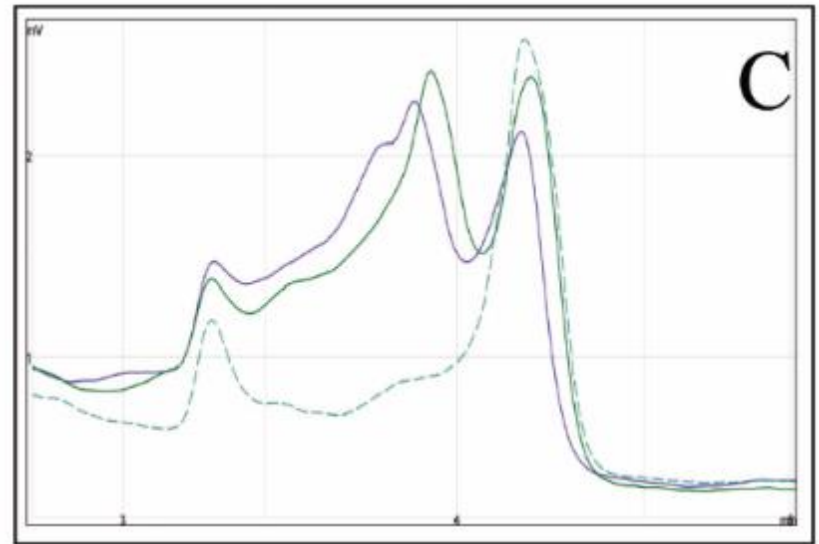
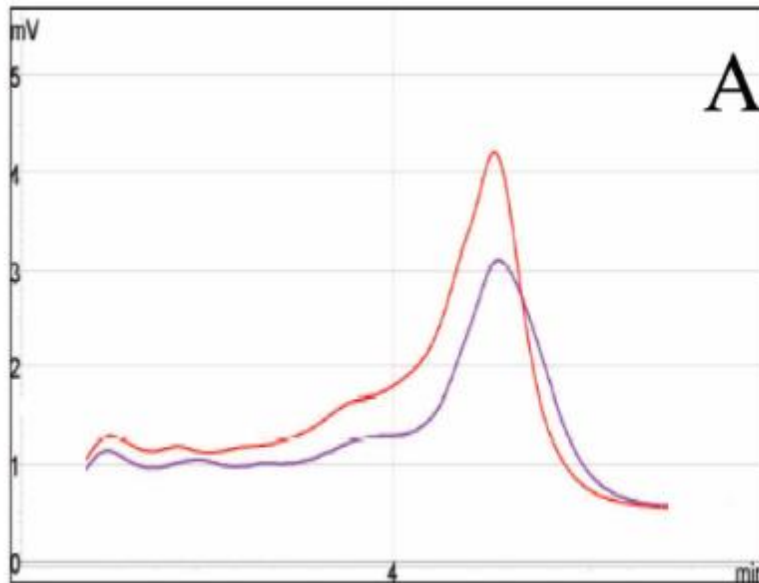
NPM1



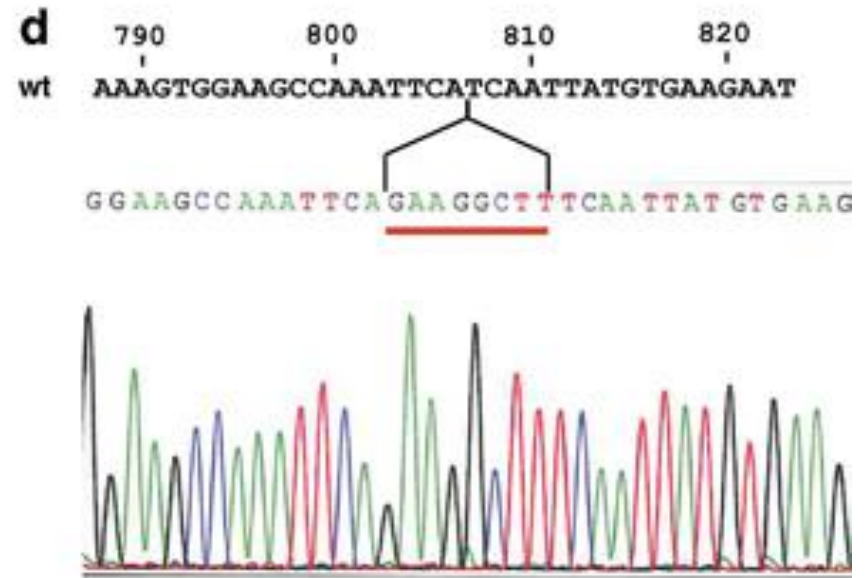
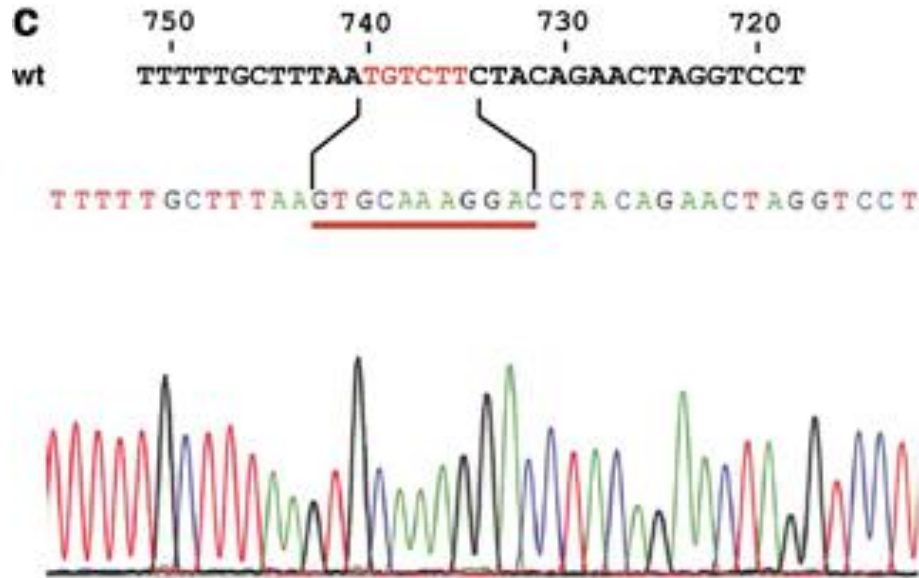
NPM1

	959																																									
Wild Type	D	L	W	Q	W	R	K	S	L	Stop	-	-	-																													
	g	a	t	c	t	c	t	g	g	c	a	g	t	g	g	a	g	g	a	a	g	t	c	t	c	t	t	t	a	a	g	a	a	a	a	t	a	g				
A	D	L	C	L	A	V	E	E	V	S	L	R	K	Stop																												
	g	a	t	c	t	c	t	g	T	C	T	G	g	c	a	g	t	g	g	a	g	g	a	a	g	t	c	t	c	t	t	t	a	a	g	a	a	a	a	t	a	g
B	D	L	C	M	A	V	E	E	V	S	L	R	K	Stop																												
	g	a	t	c	t	c	t	g	C	A	T	G	g	c	a	g	t	g	g	a	g	g	a	a	g	t	c	t	c	t	t	t	a	a	g	a	a	a	a	t	a	g
D	D	L	C	L	A	V	E	E	V	S	L	R	K	Stop																												
	g	a	t	c	t	c	t	g	C	C	T	G	g	c	a	g	t	g	g	a	g	g	a	a	g	t	c	t	c	t	t	t	a	a	g	a	a	a	a	t	a	g

DHPLC screening



NPM1



WT1

Normalmente espresso sulle cellule CD34+, richiesto per il normale sviluppo e per la sopravvivenza cellulare; poi downregolato con la differenziazione

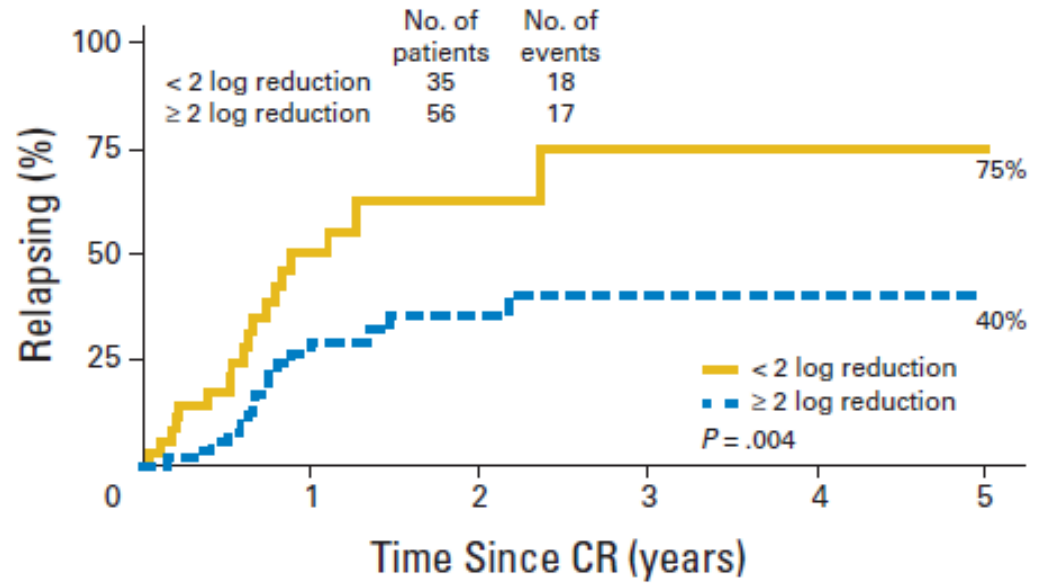
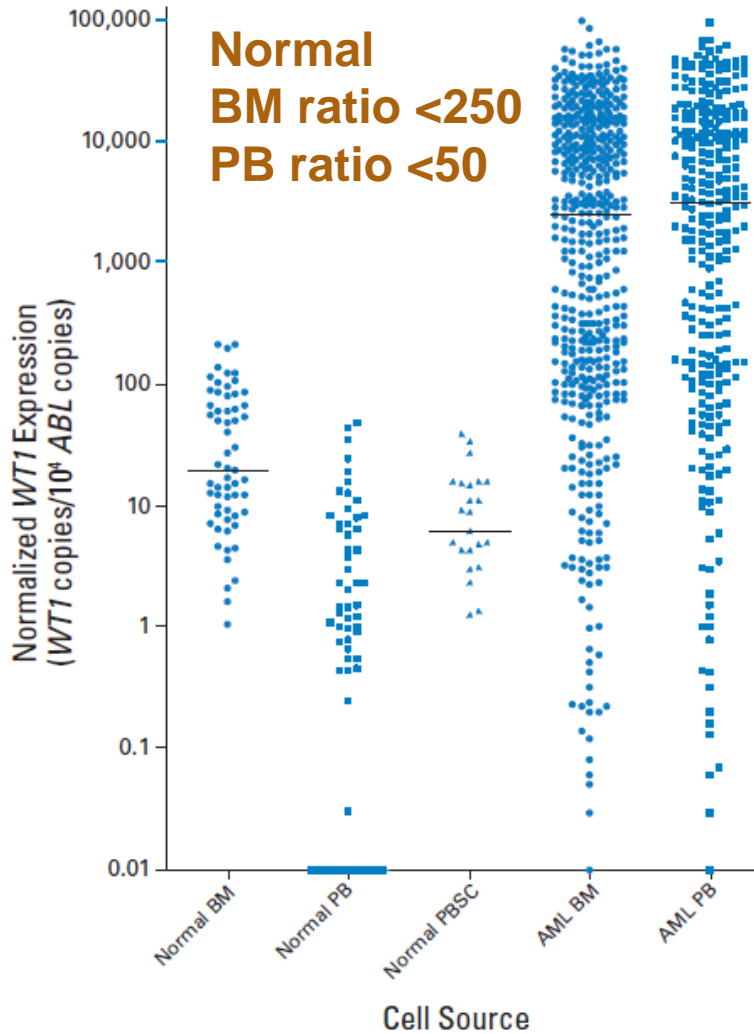


Espresso ad alti livelli sulle cellule leucemiche



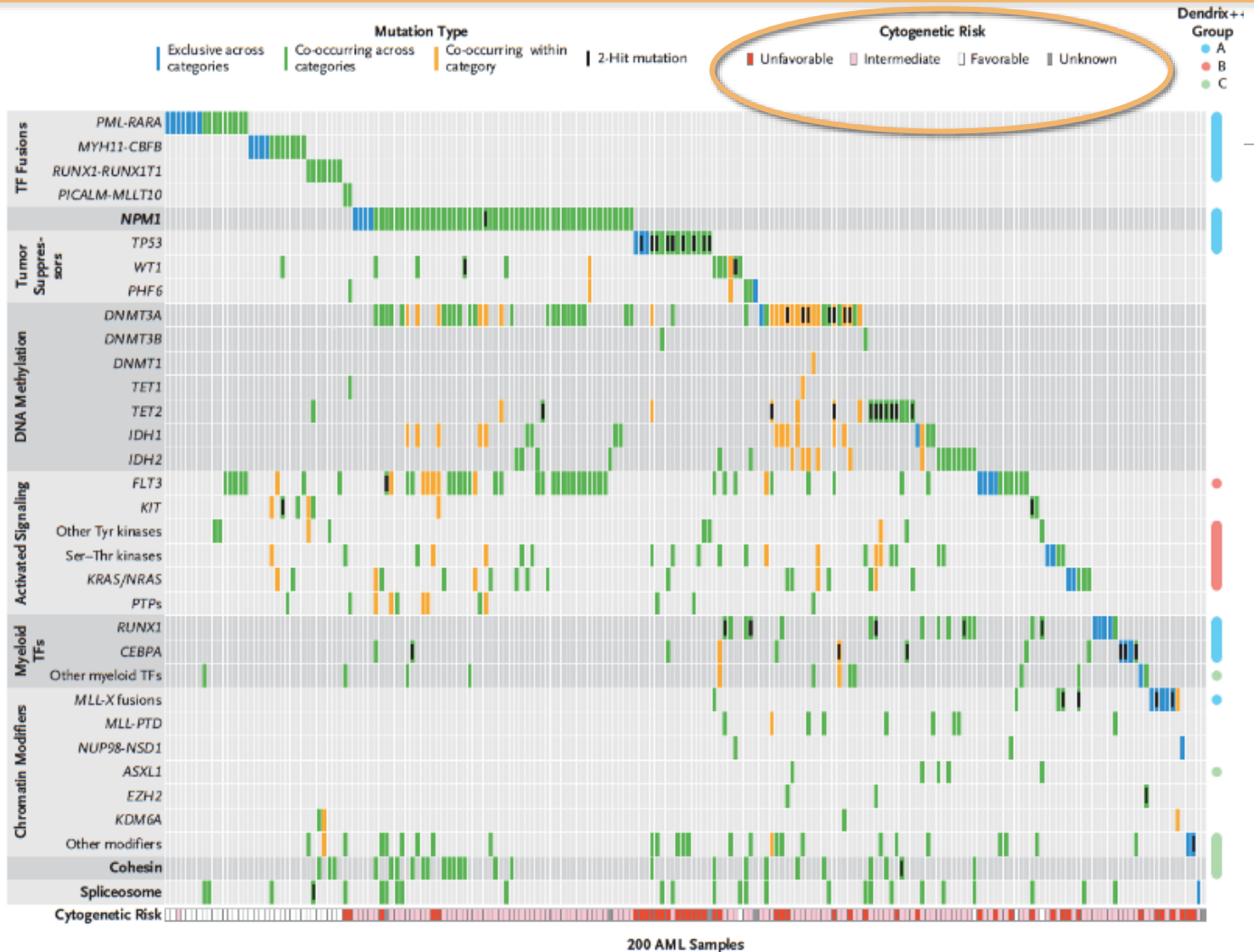
Il livello di espressione riflette la presenza di malattia minima residua

WT1

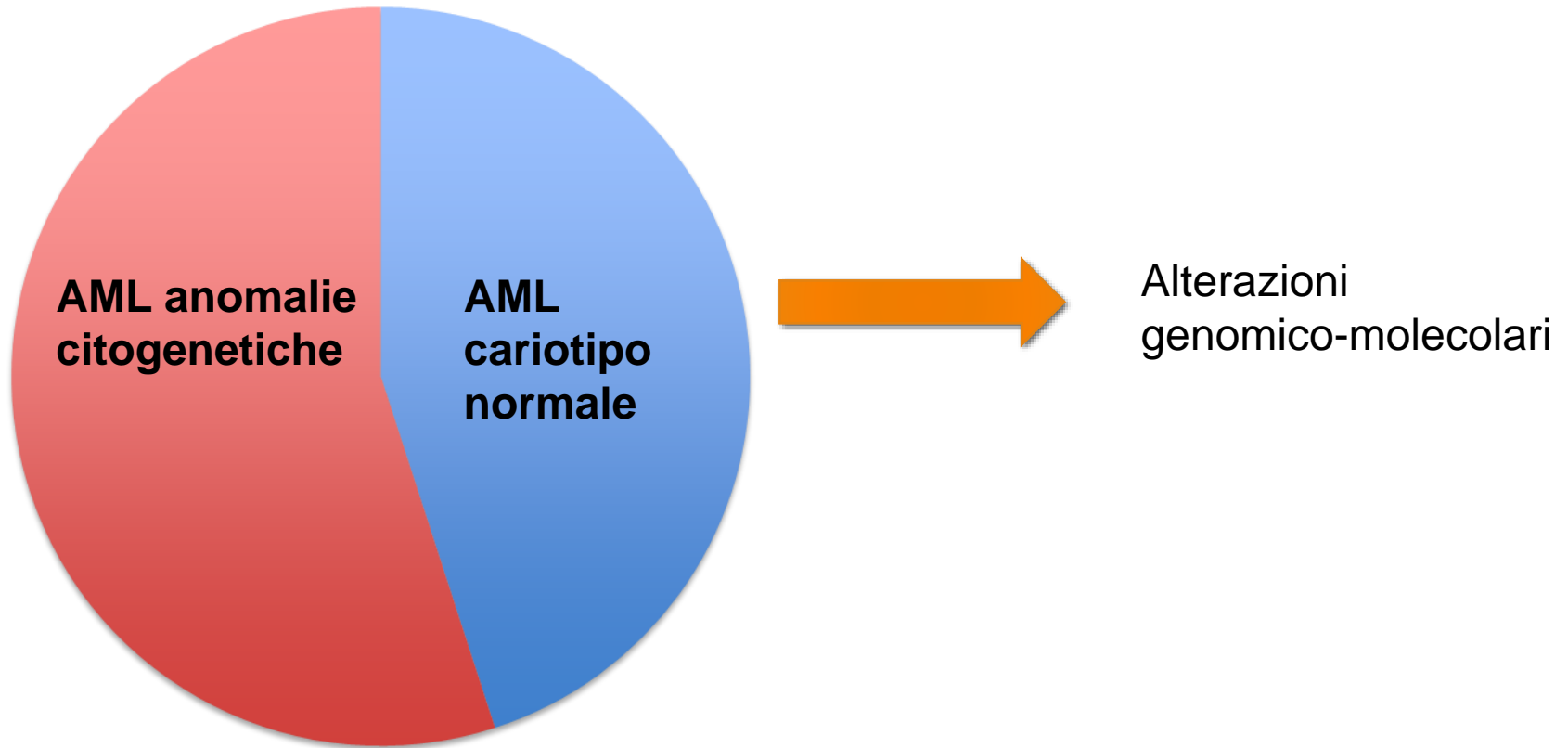


NEXT GENERATION SEQUENCING: THE INNOVATION

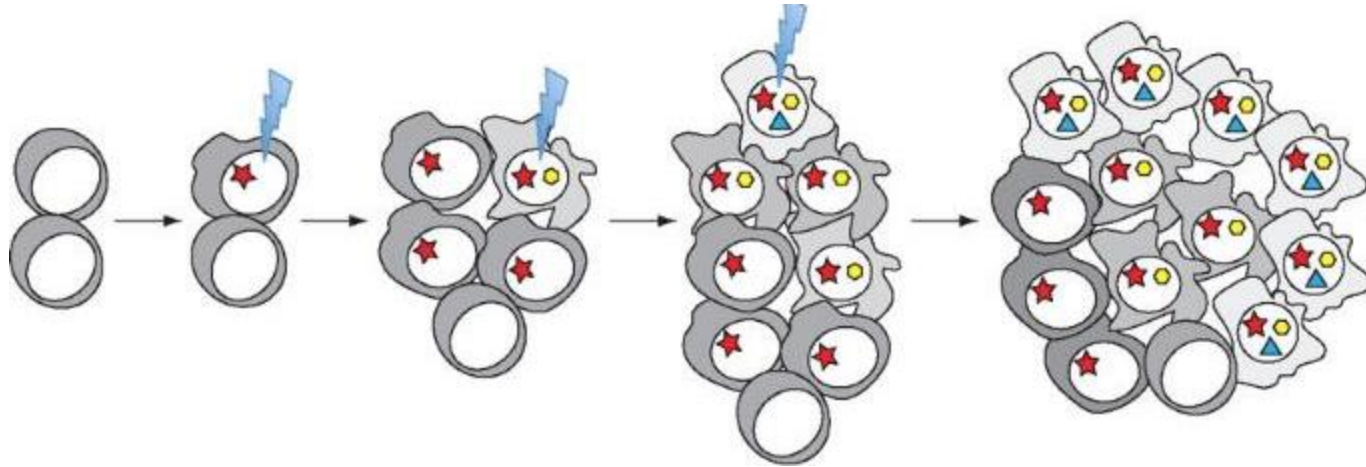
GENOMIC COMPLEXITY OF AML



GENOMIC COMPLEXITY OF AML



Leucemogenesis



L'insieme delle mutazioni fondanti e subclonali determinano:

- caratteristiche di presentazione della LAM
- aggressività
- sensibilità alle terapie

CONVENTIONAL DIAGNOSYS OF AML

Attualmente le mutazioni geniche sono studiate con metodiche convenzionali: es. Sanger

Limite di
sensibilità della
metodica Sanger

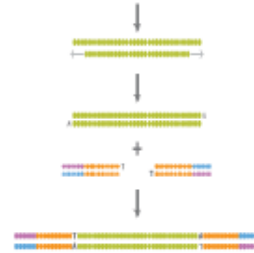


NEXT GENERATION SEQUENCING (NGS)

	Whole Genome	Enrichment	Small Genome/ Plasmid	Amplicon
Description	Basic sequencing of a genome, typically aligns to a reference sequence, can be <i>De Novo</i> sequencing, genotyping, etc.	Studies targeting the exome or planning to enrich genomic DNA for large areas of the genome	Methods optimized for handling small genomes like bacterial genomes, similar to Whole Genome methods	Either starting with PCR amplicons or using the Illumina Design Studio software to design custom amplicon probes, typically covers a smaller area of the genome than enrichment methods

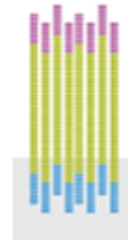
1

Library Preparation



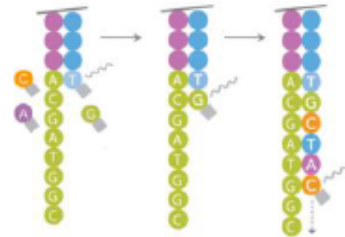
2

Cluster Generation



3

Sequencing



4

Data Analysis

```
STAAGGCTAGGTTTCATGCTA
STAAGGCTAGGTTTCATGCTA
STAAGGCTAGGTTTCATGCTA
STAAGGCTAGGTTTCATGCTA
TAAGGCTAGGTTTCATGCTA
AAGGCTAGGTTTCATGCTA
T AAGGCTAGGTTTCATGCTA
TA GCTAGGTTTCATGCTA
STA GCTAGGTTTCATGCTA
STAA CTAGGTTTCATGCTA
```

STUDY DESIGN



- traduzione del segnale
- regolatori epigenetici e trascrizionali
- splicing
- attività polimerasica
- metabolismo
- altro

TET2
DNMT3A
ASXL1
RUNX1
EZH2
BCOR
ETV6
CEBPalpha
GATA2

SF3B1
SRSF2
U2AF1
ZRSR2

ANKRD46
NRAS
KRAS
FLT3
CBL
SRP72
DDX41

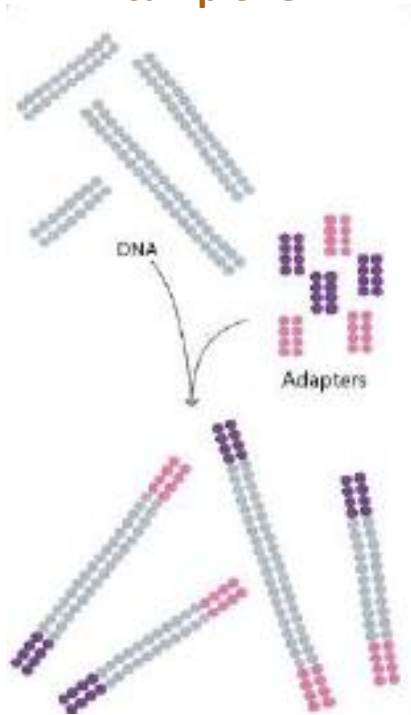
TERT
TERC

NPM1
TP53
IDH1
IDH2

Solexa - Metodology

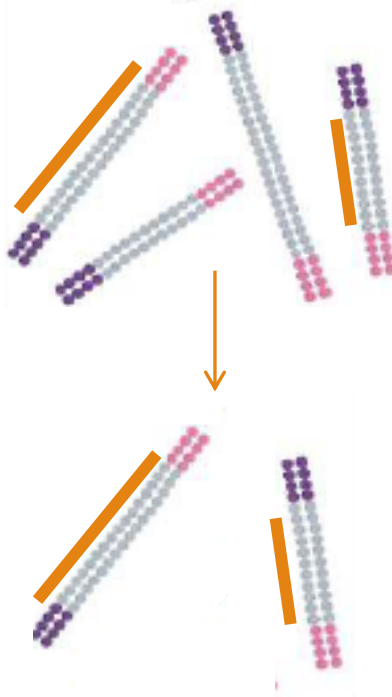
From Illumina Web Site

1. Preparazione del campione



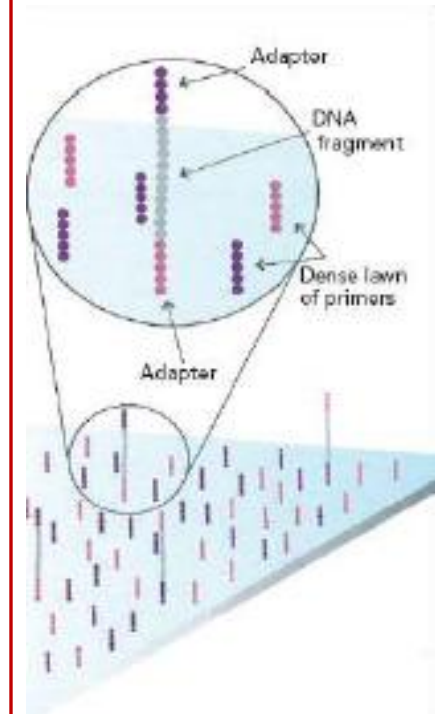
Il DNA viene frammentato e ad ogni porzione è legato un adattatore e un Index.

2. Ibridazione mediante sonde



Le porzioni di DNA di interesse vengono selezionate mediante ibridazione con sonde.

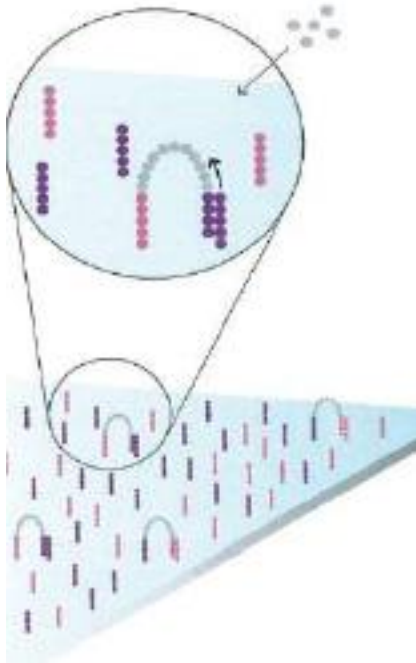
3. Legame del DNA al vetrino



Attraverso gli adattatori, i frammenti sono legati ai primer presenti sul vetrino con legame covalente

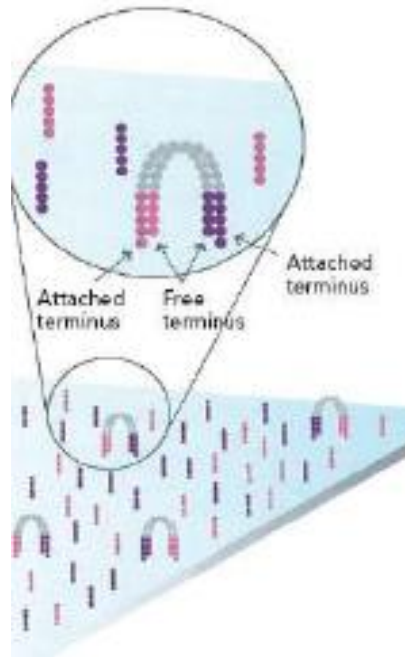
Solexa – Metodology II

4. Amplificazione “a ponte”



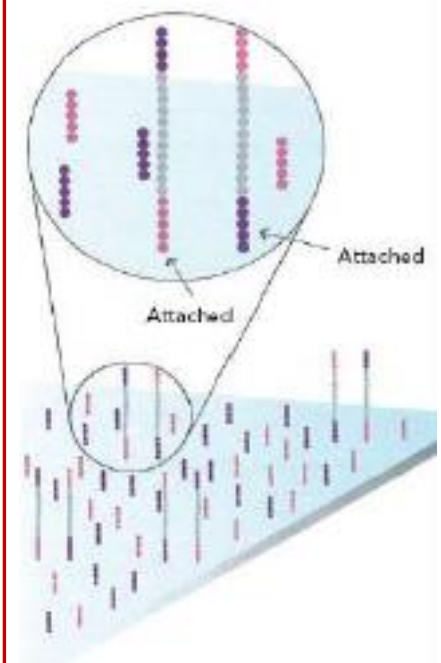
Aggiunta di nucleotidi NON marcati e amplificazione dei frammenti che formano dei ponti tra i primers.

5. I frammenti diventano a doppio strand



I ponti di DNA diventano a doppio filamento

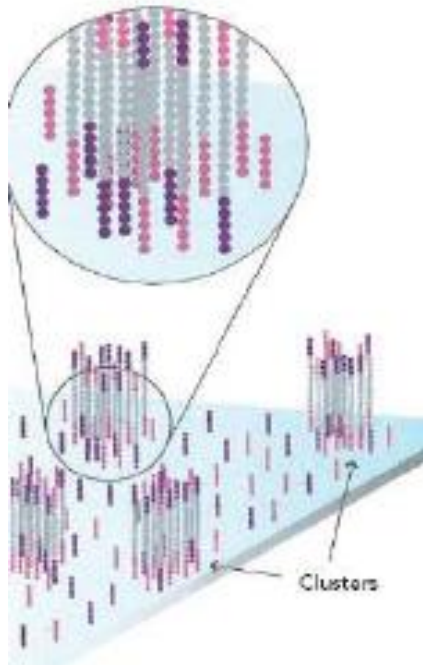
6. Denaturazione delle molecole a doppio strand



I ponti vengono linearizzati e si ottiene DNAss

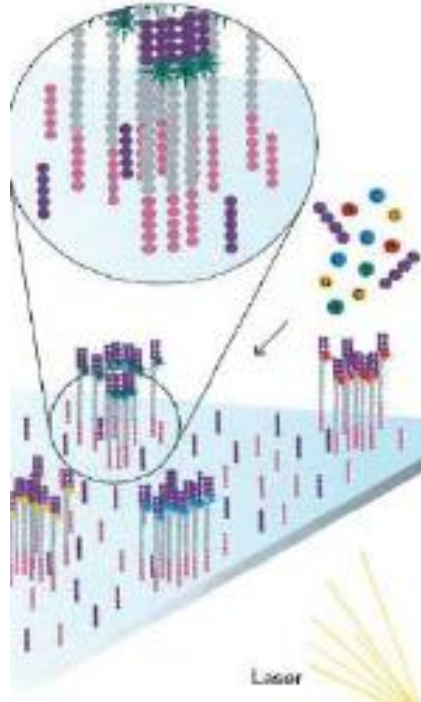
Solexa – Metodology III

7. Amplificazione



L'amplificazione crea diversi milioni di cluster di DNAs, che sono multiple copie del frammento di partenza

8. Primo ciclo di sintesi

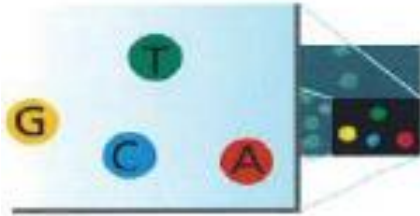


Tutti i 4 terminator reversibili sono aggiunti. Ogni base compete con le altre per il legame al template (questa competizione è la base dell'accuratezza)

Il vetrino viene poi stimolato con luce laser

Solexa – Metodology IV

9. Identificazione della prima base

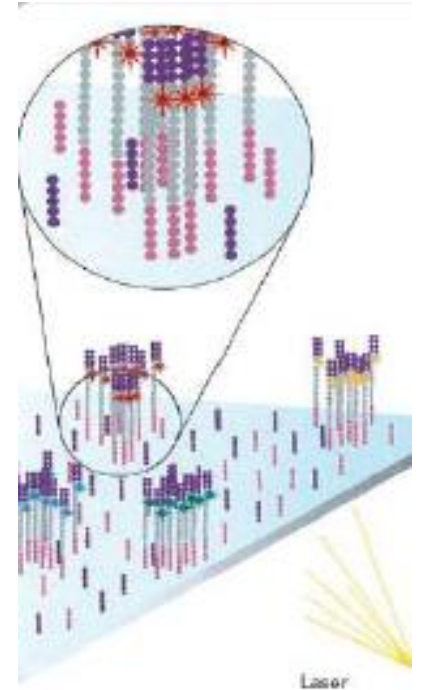


Viene catturata l'immagine della fluorescenza che origina da ogni cluster. Il colore identifica la prima base



Il gruppo fluorescente è rimosso e questo permette il legame di una nuova base

10. Identificazione delle basi successive



Ad ogni ciclo viene aggiunta e quindi identificata la base successiva.

BIOINFORMATIC ANALISYS

J. Craig Venter™
INSTITUTE

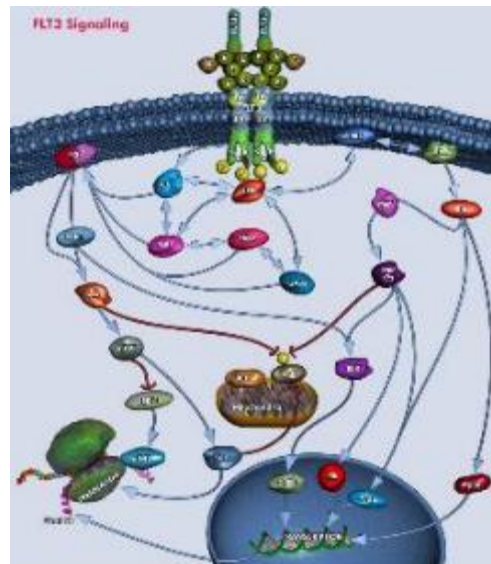
SIFT

NCBI

dbSNP
Short Genetic Variations

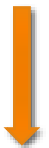


COSMIC
Catalogue of somatic mutations in cancer



ANNOTATION ANALYSIS:ANNOVAR

	A	B	C	D	E	F	G	H	I	J	K	L
1	Chr	Start	End	Ref	Alt	Func.refGe	Gene.refGene	GeneDetail.refGene	ExonicFunc.refGene	AAChange.refGene	Xref.refGene	ExAC_Freq
2	1	948921	948921	T	C	UTR5	ISG15	NM_005101:c.-33T>C	.	.	Immunodeficienc	0.941
3	1	1404001	1404001	G	T	UTR3	ATAD3C	NM_001039211:c.*91G>T	.	.	.	0.054
4	1	5935162	5935162	A	T	splicing	NPHP4	NM_001291594:exon17:c.1282-2T>A	.	.	Nephronophthisi	0.825
5	1	162736463	162736463	C	T	intronic	DDR2	.	.	.	Spondylometaepi	.
6	1	84875173	84875173	C	T	intronic	DNASE2B
7	1	13211293	13211294	TC	-	intergenic	PRAMEF36P;F	dist=11566;dist=116902
8	1	11403596	11403596	-	AT	intergenic	UBIAD1;PTCH	dist=55105;dist=135699
9	1	105492231	105492231	A	ATAAA	intergenic	LOC10012913	dist=872538;dist=640085
10	1	67705958	67705958	G	A	exonic	IL23R	.	nonsynonymous SNV	IL23R:NM_144701:exon9:c.G1142A:p.R381Q	.	0.041
11	2	234183368	234183368	A	G	exonic	ATG16L1	.	nonsynonymous SNV	ATG16L1:NM_198890:exon5:c.A409G:p.T137A;ATG16L1:NM_0	.	0.457
12	16	50745926	50745926	C	T	exonic	NOD2	.	nonsynonymous SNV	NOD2:NM_001293557:exon3:c.C2023T:p.R675W;NOD2:NM_0	Blau syndrome, A	0.023
13	16	50756540	50756540	G	C	exonic	NOD2	.	nonsynonymous SNV	NOD2:NM_001293557:exon7:c.G2641C:p.G881R;NOD2:NM_0	Blau syndrome, A	0.009917
14	16	50763778	50763778	-	C	exonic	NOD2	.	frameshift insertion	NOD2:NM_001293557:exon10:c.2936dupC:p.L980Pfs*2;NOD2	Blau syndrome, A	0.013
15	13	20763686	20763686	G	-	exonic	GJB2	.	frameshift deletion	GJB2:NM_004004:exon2:c.35delG:p.G12Vfs*2	Bart-Pumphrey sy	0.006038
16	13	20797176	21105944	O	-	exonic	CRYL1;GJB6	.	frameshift deletion	GJB6:NM_001110220:wholegene;GJB6:NM_001110221:whole	.	.
17	8	8887543	8887543	A	T	exonic	ERI1	.	stoploss	ERI1:NM_153332:exon7:c.A1049T:p.X350L	.	.
18	8	8887539	8887539	A	T	exonic	ERI1	.	stopgain	ERI1:NM_153332:exon7:c.A1045T:p.K349X	.	.
19	8	8887536	8887537	AG	GATT	exonic	ERI1	.	frameshift substitutic	ERI1:NM_153332:exon7:c.1042_1043GATT:p.R348Dfs*2	.	.
20	8	8887540	8887540	G	GGAA	exonic	ERI1	.	nonframeshift substit	ERI1:NM_153332:exon7:c.1046delinsGGAA:p.R348_K349insR	.	.
21	5	1295288	1295288	G	A	upstream	TERT	dist=126
22	chr14	95602958	95602958	A	C	splicing	DICER1	NM_001271282:exon1:UTR5	.	.	Goiter, multinodi	.



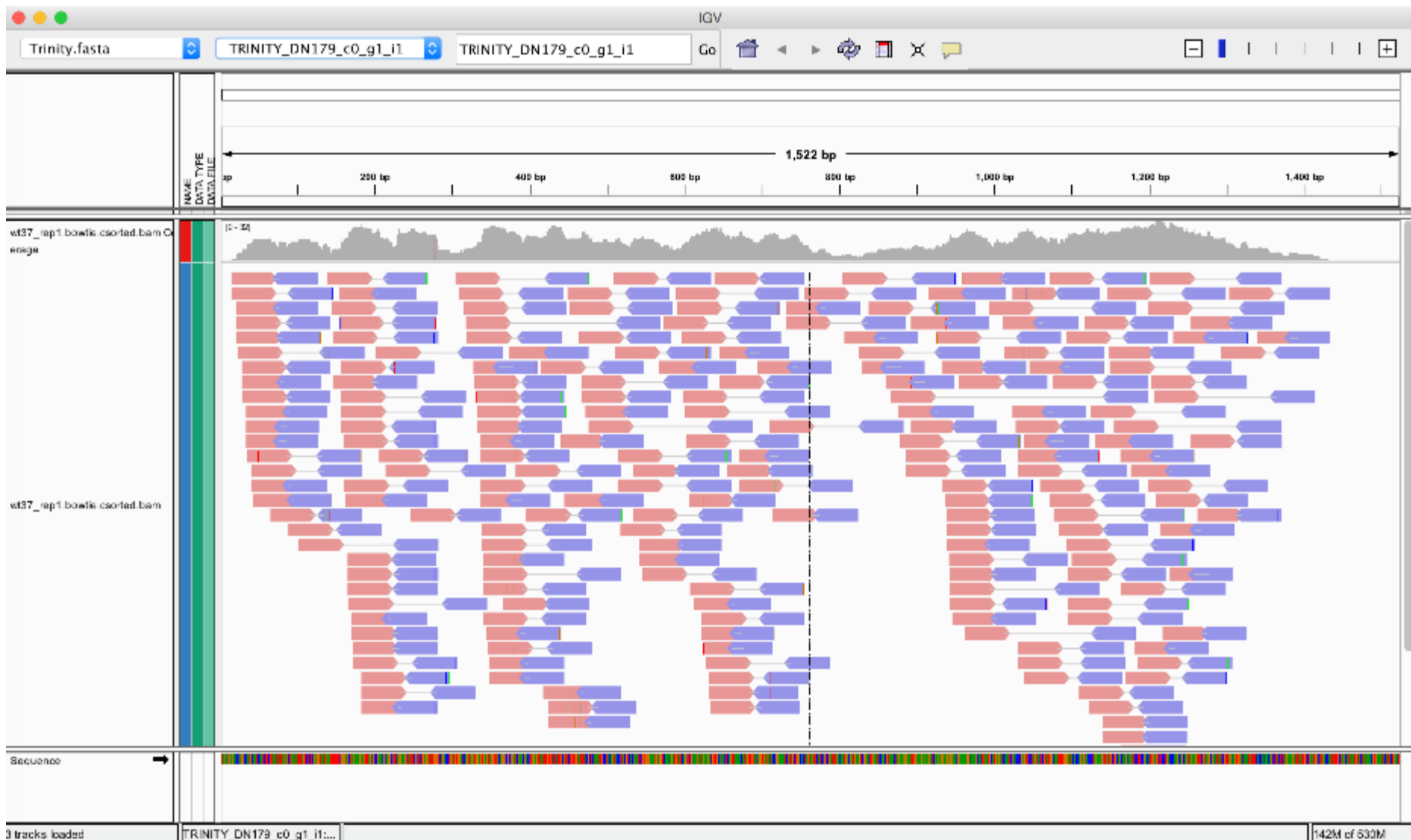
POSITION AND
SEQUENCE
ALTERATION

EFFECTS ON THE
PROTEIN

ALTERATION OF
ISOFORMS

DATABASE AND
PREDICTION
SOFTWARES

SECONDARY ANALYSIS: IGV



A.I.: e.g. SOPHIA GENETICS

Sophia DDM

DASHBOARD WORKSPACE SC10000001 / #2-0336 Requests

Sophia Genetics SA

analysis #26027 SC10000001 / MID S1 -- request: #2-0336 21/01/13 Illumina_MR_BRCA_CNV Jurgi Camblong

BRCA 2 genes 1

REPORTED 1 BRCA1 BRCA MASTR™ Dx germline

OVERVIEW SCREENING GENES SNPs-INDELS CNVs WARNINGS

Variant List - sorted by: PRED_CAT > PATHOGENICITY_CLASS > GENE

P...	Pat	id	type	cod. cons.	gene	refSeqid	c.DNA	Protein	VF%	refSeq	altSeq	depth	SC
C	5	29	SNP	intronic	BRCA1	NM_007294	c.4485-63C>G		49.87			770	
C	5	27	SNP	intronic	BRCA1	NM_007294	c.4987-68A>G		53.63			716	
C	4	2	SNP	missense	BRCA1	NM_007294	c.2077G>A	p.Asp693Asn	51.43	GAC	AAC	525	
C	2	33	SNP	5'UTR	BRCA2	NM_000059	c.-26G>A		50.0			1020	
C	1	25	SNP	intronic	BRCA1	NM_007294	c.5152+66G>A		51.94			258	
C	1	18	INDEL	intronic	BRCA2	NM_000059	c.6841+80...		51.14			1095	
C		36	SNP	5'UTR	BRCA1	NM_007294	c.-134T>C		55.74			540	
C		26	SNP	intronic	BRCA1	NM_007294	c.5075-53C>T		55.41			231	
C		32	INDEL	intronic	BRCA1	NM_007294	c.548-58delT		51.32			793	
C		31	SNP	intronic	BRCA1	NM_007294	c.4097-141A...		46.97			264	
C		28	SNP	intronic	BRCA1	NM_007294	c.4987-92A>G		53.63			716	

Static Filters: Retained 24, A: Most Likely Patho... 2, B: Potentially Patho... 1, C: VUS (Unknown ... 3, D: Likely Benign 3, Low Confidence 3, Flagged 11

OVERVIEW DETAILS COMMENTS VIEWER SIMILAR PATIENTS WARNINGS

reads: 716 DEPTH, 231 min, 6491 max

frequencies: 4/11 RUN, 24% ACCOUNT, 53.4% COMMUNITY

flagging: 13, 5, In Report 2, Set To False + 0

transcript: NM_007294, cDNA: c.4987-68A>G, refSeq: T->C, rs8176234

SNP: 17-16, intronic

SNP BRCA1: ExAC, ClinVar, COSMIC, IGV, NCBI, ALAMUT

PolyPhen2: ns, SIFT: ns, MutationTester: ns, ESP5400: 0.0, ExAC: 0.0, cg68: 0.26, G1000: 0.35

Values are scaled so that the most pathogenic scores are plotted towards the external circle. ESP5400 & G1000 empty values are considered as 0.0

Sophia Genetics SA 05-1-2016 02:21:31 A, O 3.5.0.5-r7411