# Ageing men, selfish testes and paternal age-effect mutations

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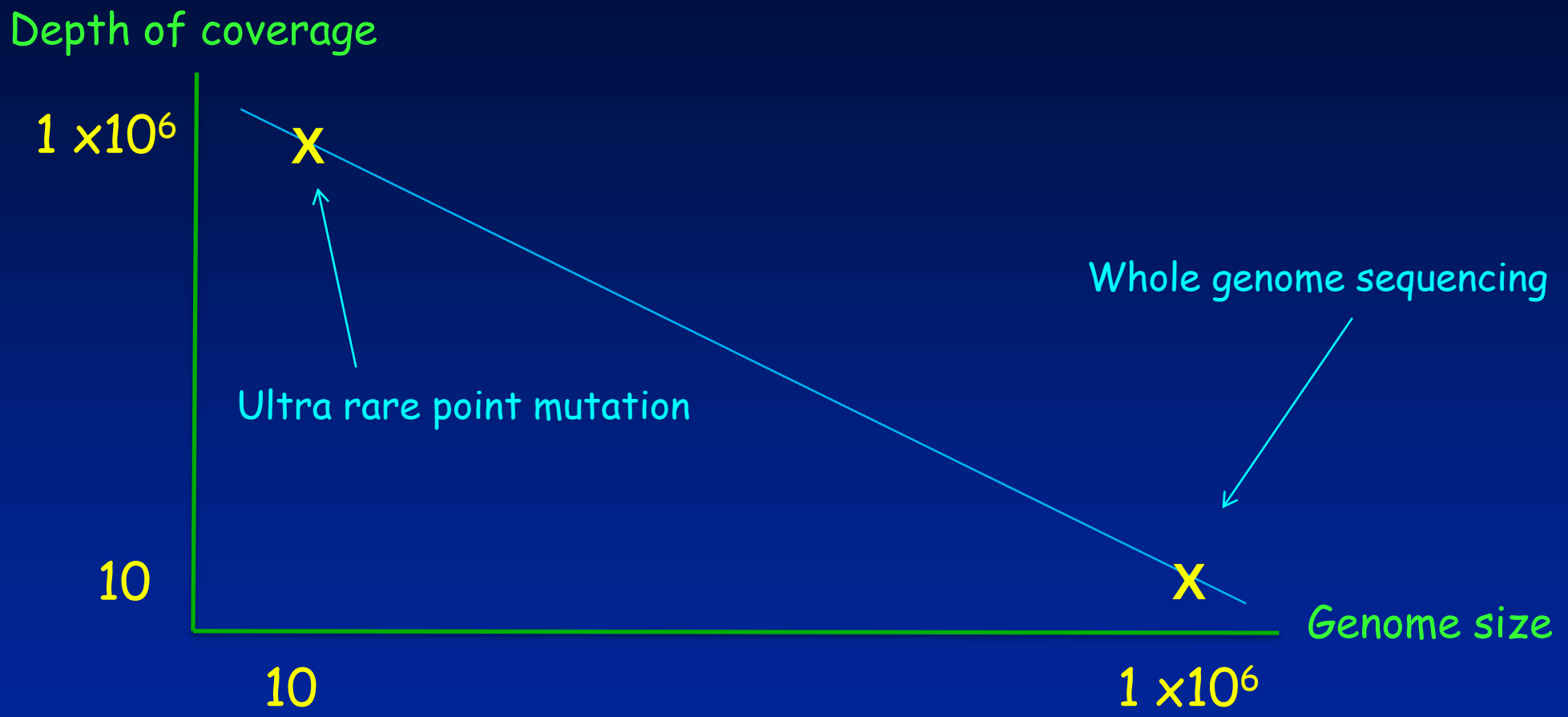
# Tailoring Next-Gen Sequencing throughput




Study of Paternal Age-Effect mutations in human sperm

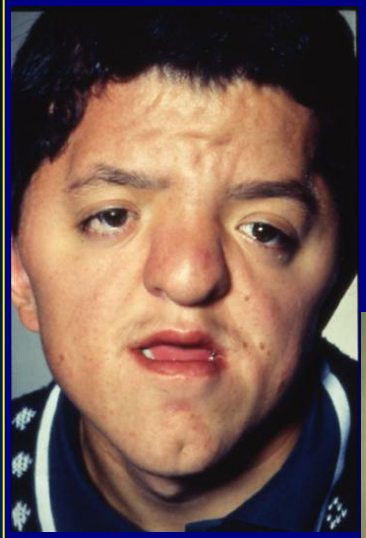
# "Paternal age-effect" mutations and associated disorders

Spontaneous dominant disorders
Specific point mutations (GOF mutations)
100-1000x more common than background
Exclusive (or near-) paternal origin and
Paternal age-effect (~ 2-5 years older than average)

1. *FGFR2* (Apert syndrome, Pfeiffer syndrome, Crouzon syndrome)
2. *FGFR3* (achondroplasia, thanatophoric dysplasia, Muenke syndrome)
3. *HRAS* (Costello syndrome)

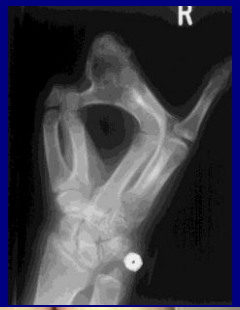
*PTPN11* (Noonan syndrome)
*RET* (Men2a,2b)

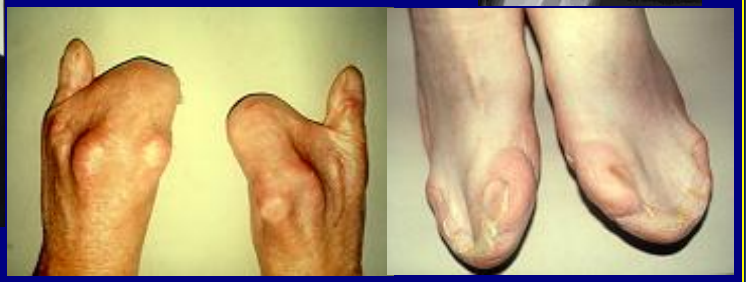
# Clinical features of Apert Syndrome



**Craniosynostosis**

(premature fusion of cranial sutures)

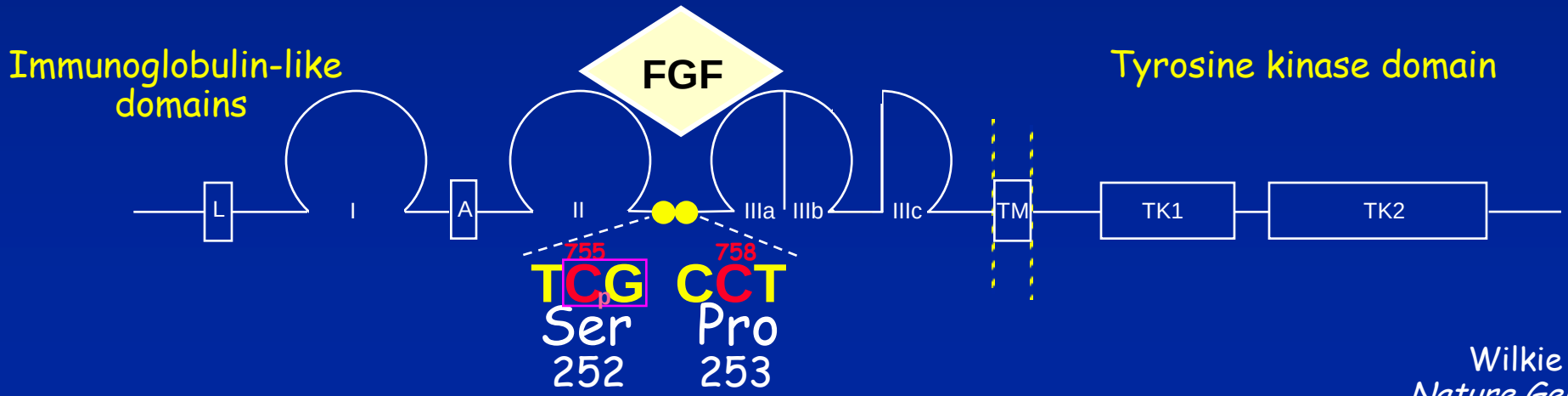




Severe syndactyly of hands and feet

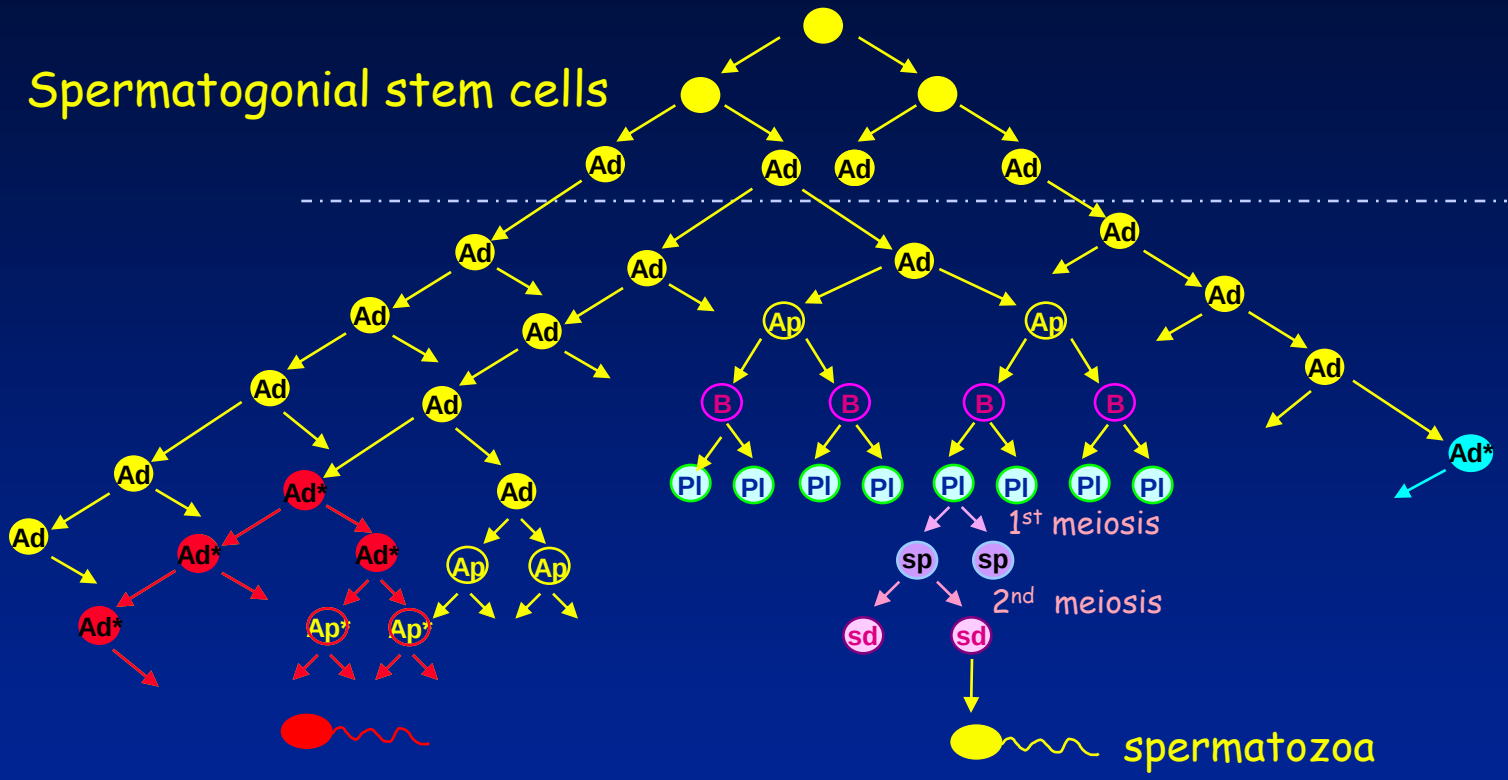
- Autosomal dominant
- > 98% cases occur by *de novo* mutation
- Birth prevalence: 1 in 65,000
- 99% of cases are caused by one of 2 specific nucleotide transversions in *FGFR2*
  - 66%: 755C>G (Ser252Trp)
  - 33%: 758C>G (Pro253Arg)
- 755C>G spontaneous mutation rate: 1:100,000
  - = ~ 1000-fold higher than background
- Paternal age effect and exclusive paternal origin
  - → Mutation occurs during spermatogenesis

## Fibroblast Growth Factor Receptor 2 structure




Wilkie *et al*, *Nature Genet* (1995)

# Human spermatogenesis and copy-error hypothesis




~~Hypothesis:~~

- mutations accumulate through multiple replication errors
- these errors increase in frequency with age

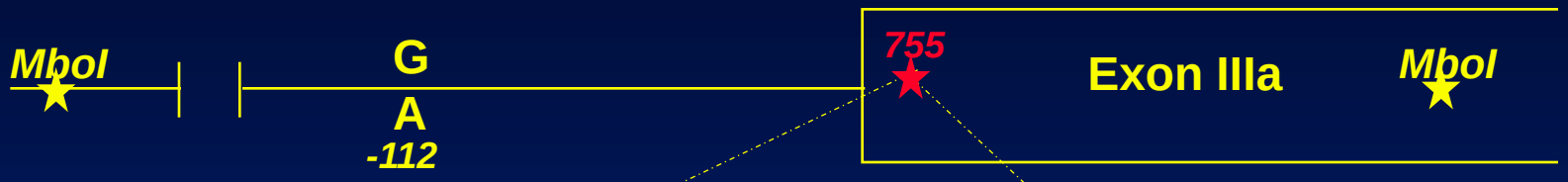
Positive selection of mutant stem cells/progenitors

# 1999-2000: Looking at the Apert mutation levels directly in sperm DNA?

- Can we find the Apert mutations in sperm?
  → Levels anticipated to be around 1:100,000 (755C>G)

- Why are the Apert mutations so 'frequent'?
  Is it a unique case?

# Selection of mutant sequences at *FGFR2* position 755 by *MboI* digestion




wt digestion: 543bp + 118bp
Mutant: 661bp *MboI*-resistant fragment

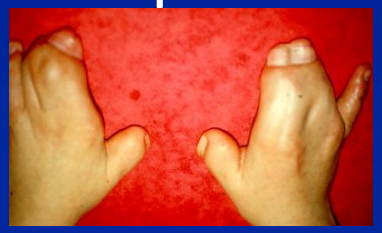
*MboI* *755*

**CGA TCG CCT CAC CGG**

| Arg | Ser | Pro | His | Arg |
|---|---|---|---|---|
| 251 | 252 | 253 | 254 | 255 |

*755*

**TGG** Trp — Apert



**TAG** Stop

**TTG** Leu

**TCG** Ser — wt

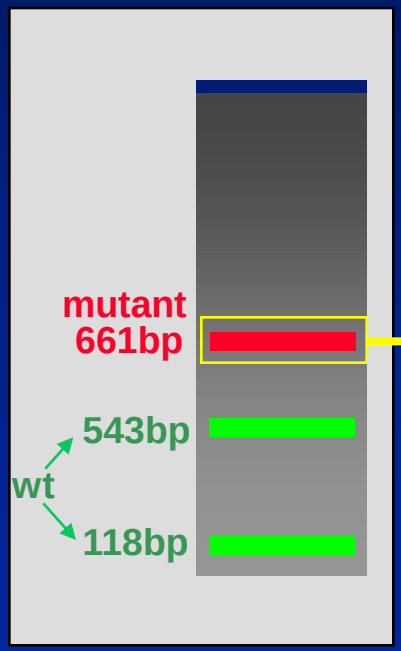
**TTC TCT** Phe Ser — spike (GR)

Goriely *et al* Science (2003)

# Quantifying Apert mutations in sperm

Genomic DNA + Spike DNA

*MboI* digestion




PCR amplification of *MboI*-resistant fragment

*MboI* digestion

2nd set of PCR with nested primers

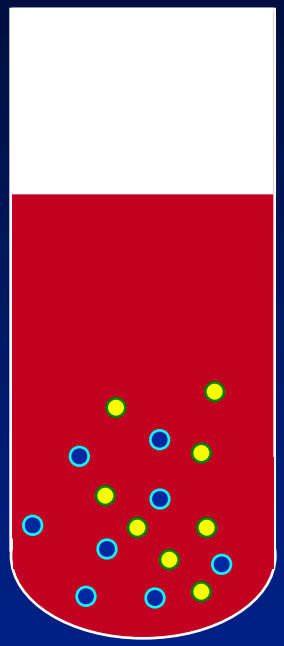
Pyrosequencing™

Greatly enriched mixture of MboI-resistant fragments

How to quantify it??

Goriely *et al* Science (2003)

# Reconstitution experiment



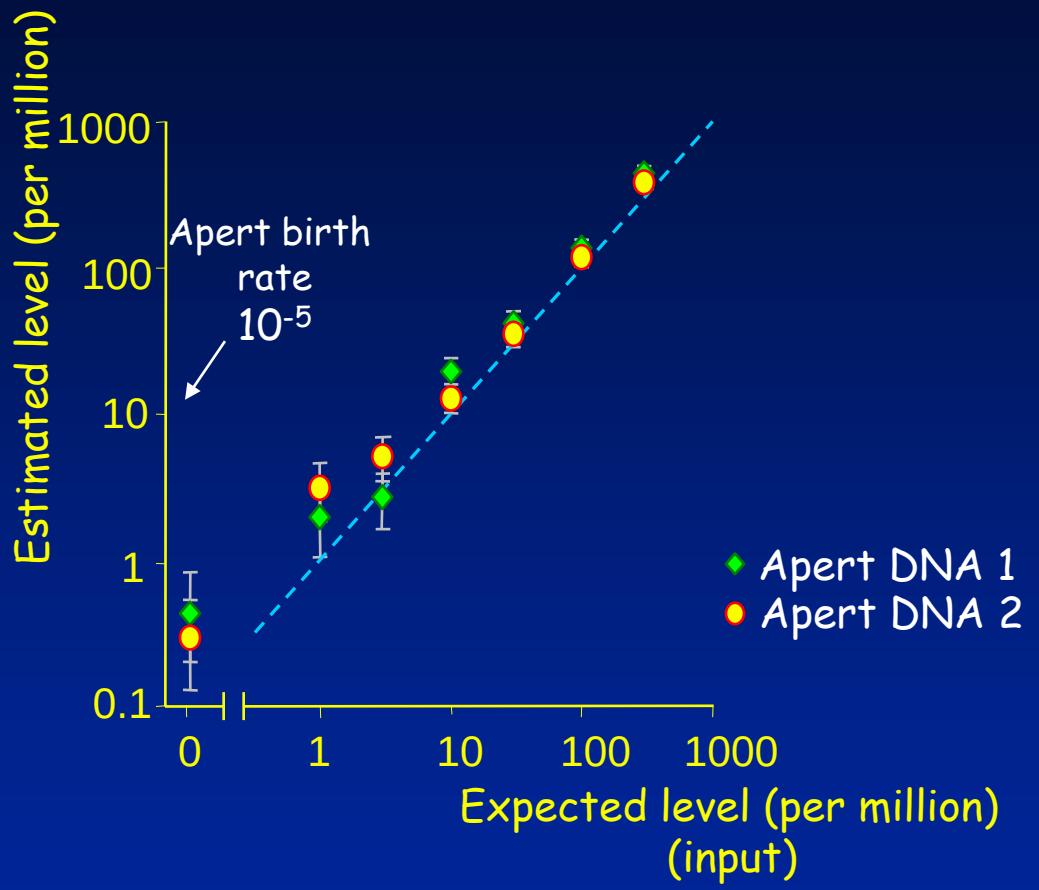
10 µg of blood DNA

+

- 755 Apert genomic DNA
  $10^{-6}$ -> $3.10^{-4}$

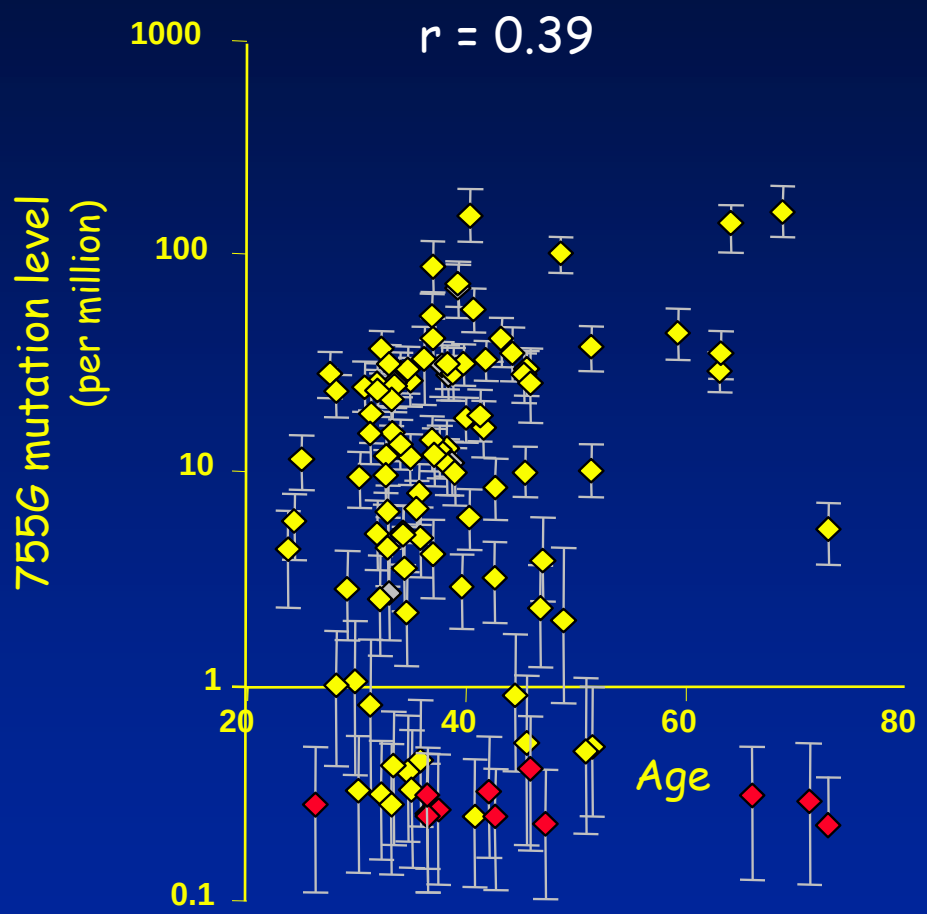
±

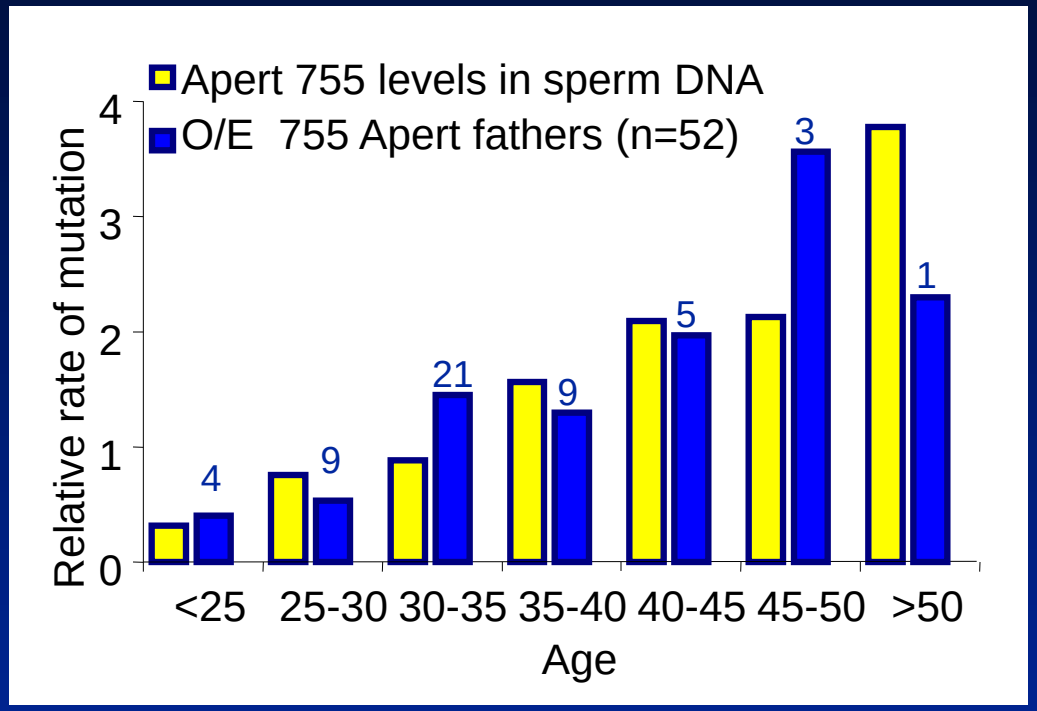
- Spike DNA (GR triple mutant)




Pyrosequencing adequately quantifies Apert mutation

# Apert mutation levels at position 755



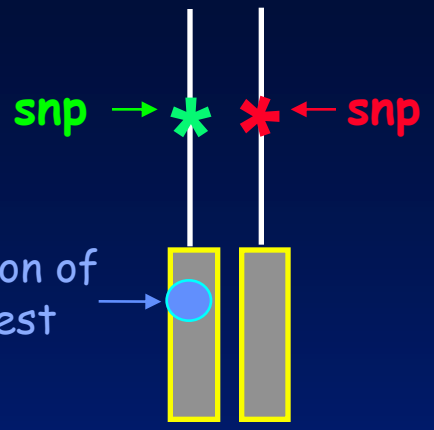




Paternal age effect is explained by the levels of 755C>G mutations in sperm of normal men

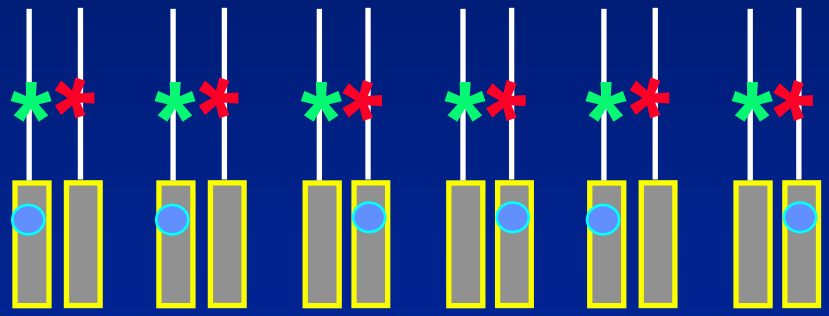
Why is Apert mutation so 'common'?

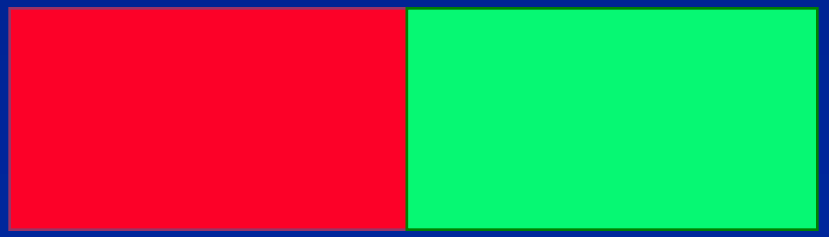
Goriely *et al Science* (2003)

# Copy-error hypothesis *vs.* selection?



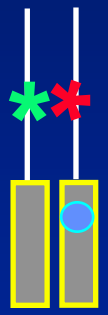

Neutral model (copy-error)

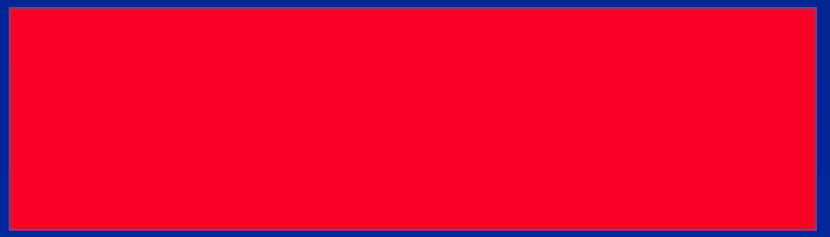




50:50 distribution of the snp

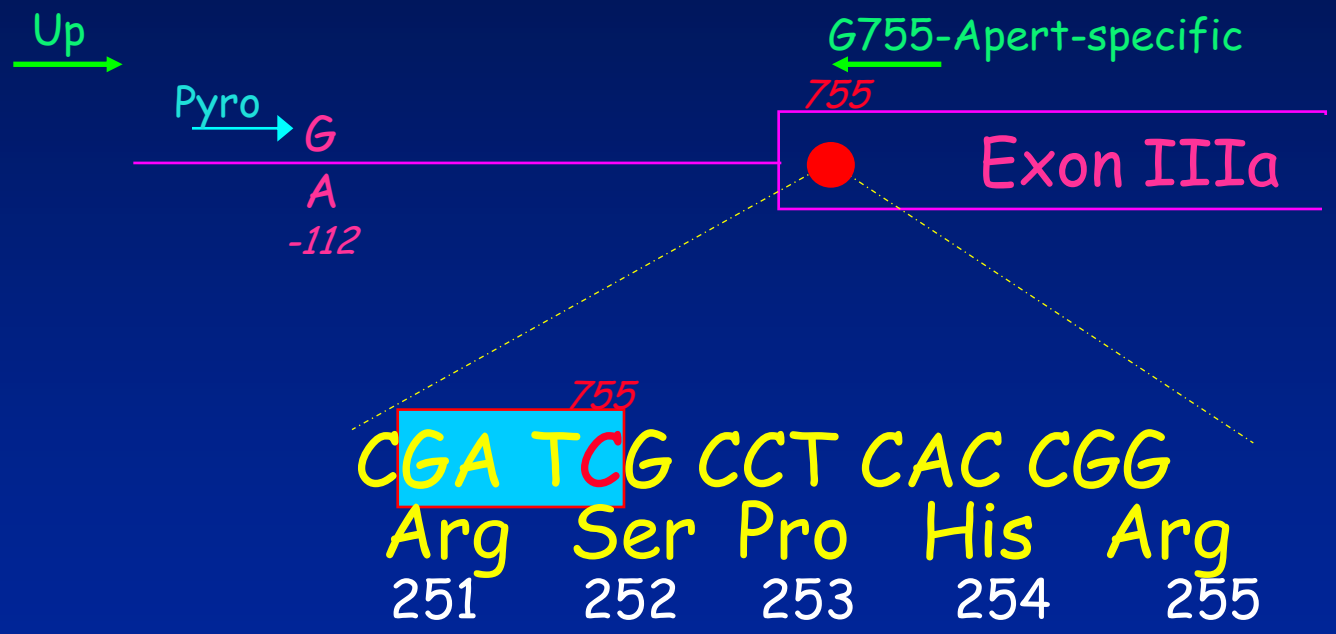
Selection model





Skew of the snp distribution

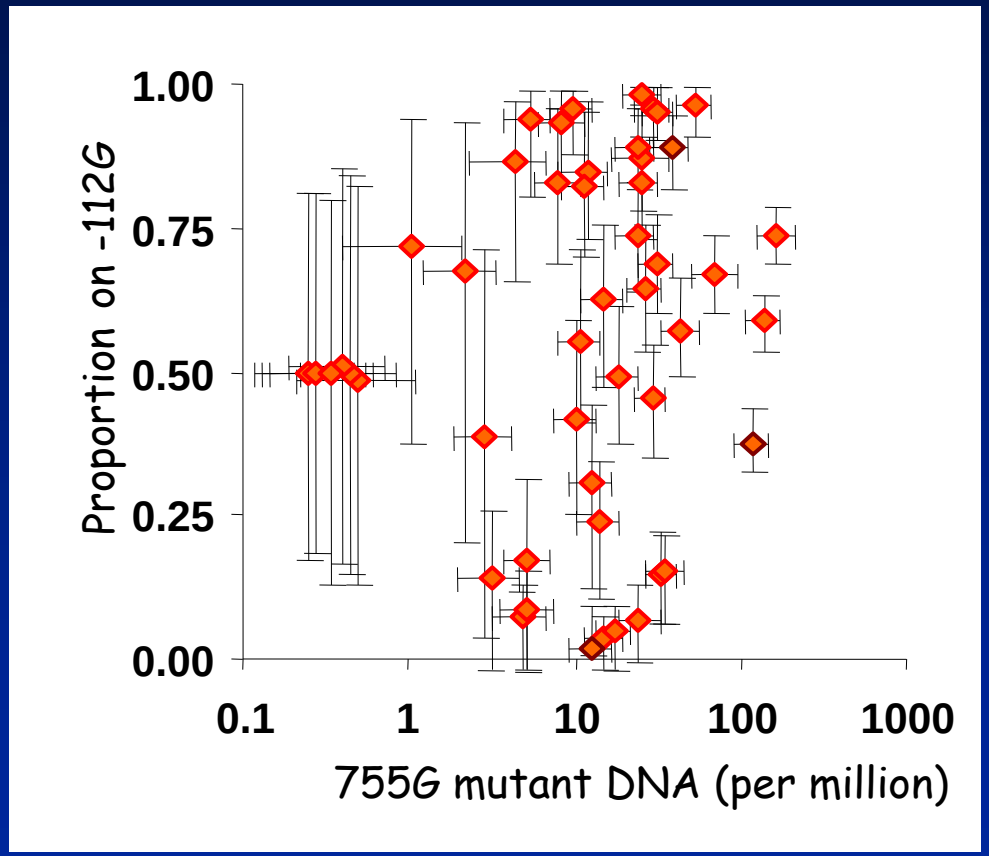
# Distribution of the 755 C>G mutant alleles in respect to -112 G/A snp

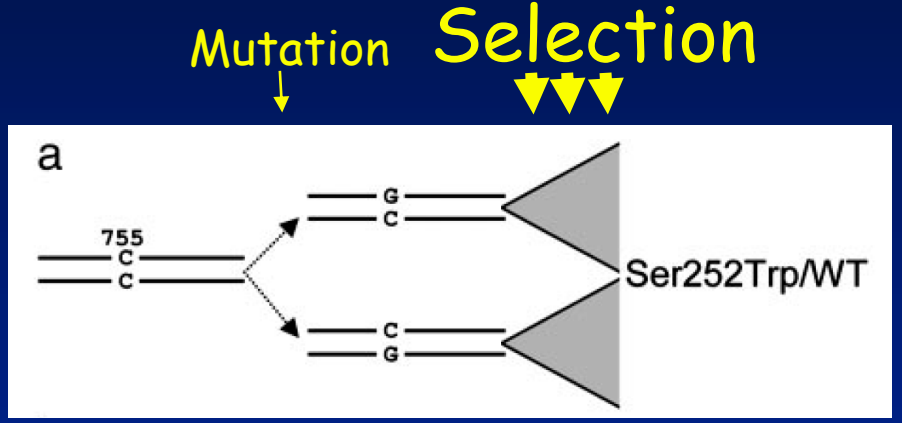



(n = 46)

# Unequal distribution of FGFR2 alleles provides evidence for selection

755 C>G Apert



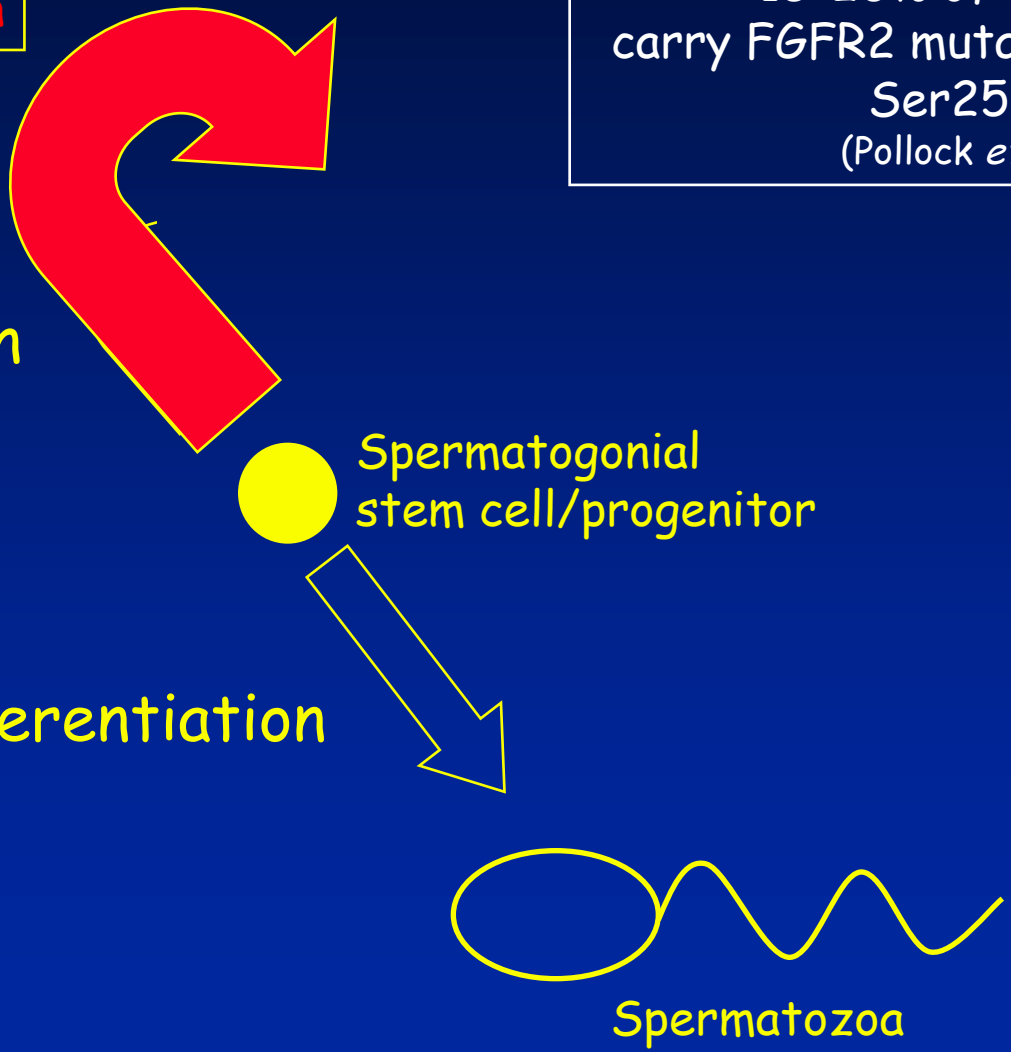

Mutational events are infrequent but confer a selective advantage to the mutant spermatogonial stem cells

Goriely *et al Science* (2003), *PNAS* (2005)

# Proposed mechanism

FGFR2
Ser252Trp
Apert mutation

15-20% of endometrial cancers carry FGFR2 mutations (half have the Apert Ser252Trp mutation)
(Pollock *et al.*, *Oncogene* 2007)

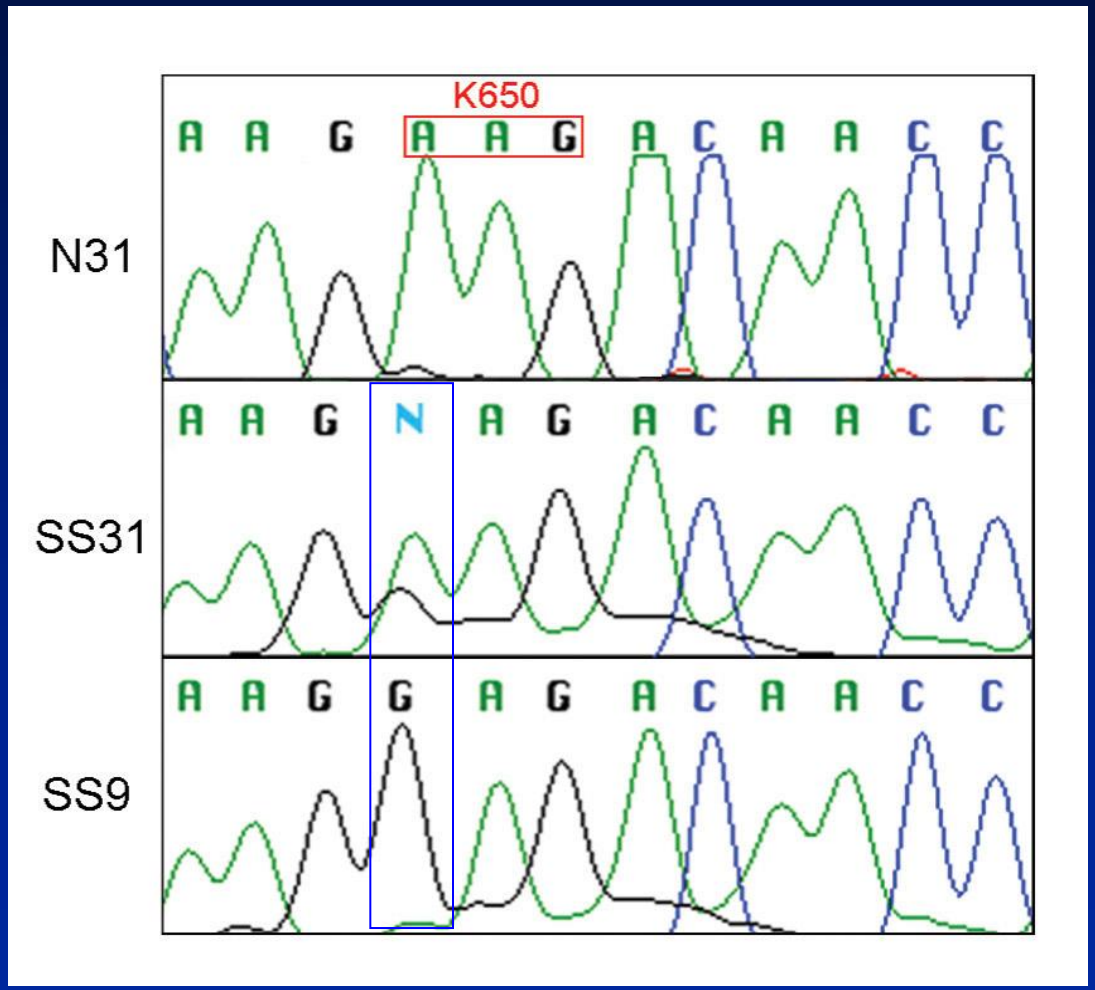



Clonal expansion of FGFR2 mutant spermatogonial cells

Testicular tumours?

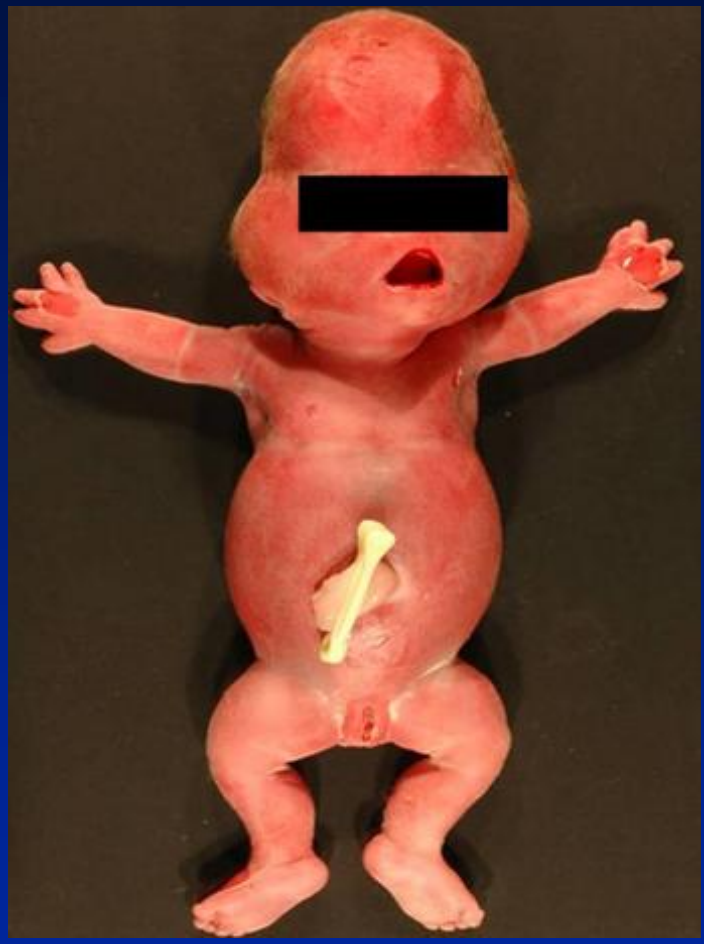
# FGFR3 mutation in testicular tumours

1948A>G (K650E) mutation in *FGFR3*




n = 30 spermatocytic seminoma

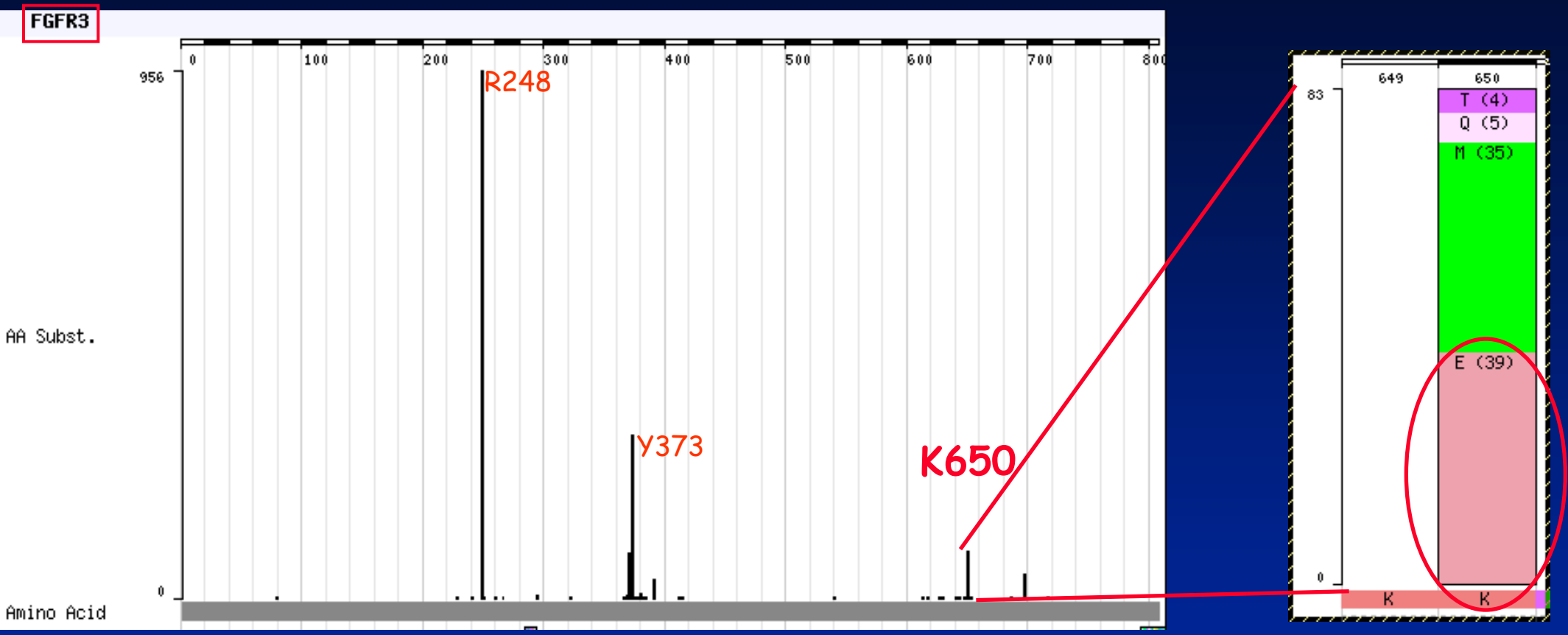
Goriely *et al*, *Nat Genet*, 2009



Dighe *et al Radiographics*, 2008

Thanatophoric Dysplasia (TD II)

# FGFR3 TD mutations in cancer




COSMIC Catalogue Of Somatic Mutations In Cancer

Biochemically: GOF = ligand-independent constitutive activation of FGFR3

Bladder carcinoma (TCC)
Seborrheic keratoses
Multiple myeloma

*www.sanger.ac.uk/genetics/CGP/**cosmic**/*

# Clinical genetics of *FGFR3* K650 codon

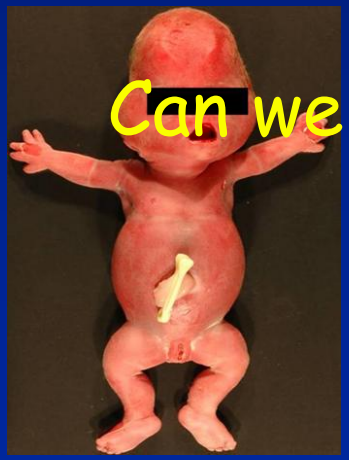
K650

AAG AAG ACA ACC

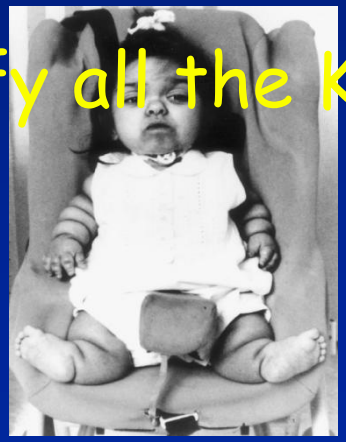
1948 1949 1950 *Bbs* I

| | | |
|---|---|---|
| A>C K650Q HCH1 | A>C K650T Fam. AN | G>A K650K silent |
| A>G K650E TDII | A>G K650R ? | G>C K650N HCH2 |
| A>T K650Ter | A>T K650M SADDAN | G>T K650N HCH3 |

Can we quantify all the K650 mutations in sperm?

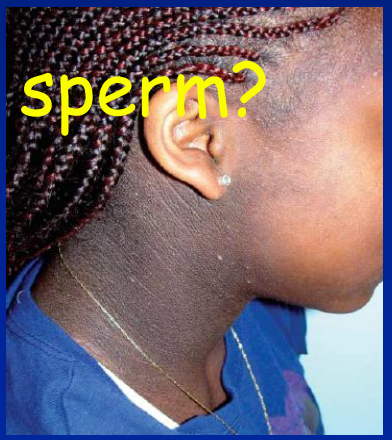


**TD II**
(Dighe *et al Radiographics* 08)



SADDAN
(Tavormina *et al AJHG* 99)

Hypochondroplasia (HCH)
(Bellus *et al AJHG* 00)

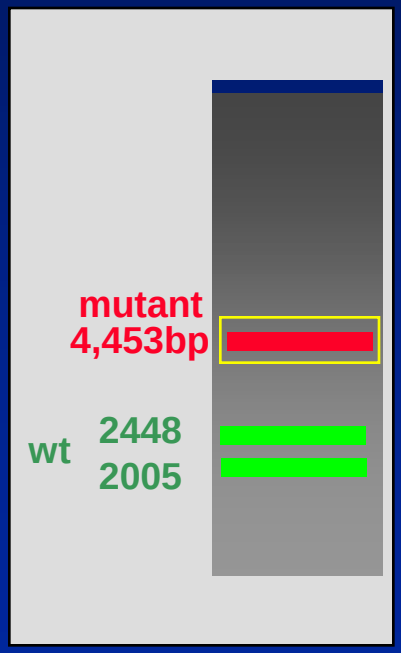


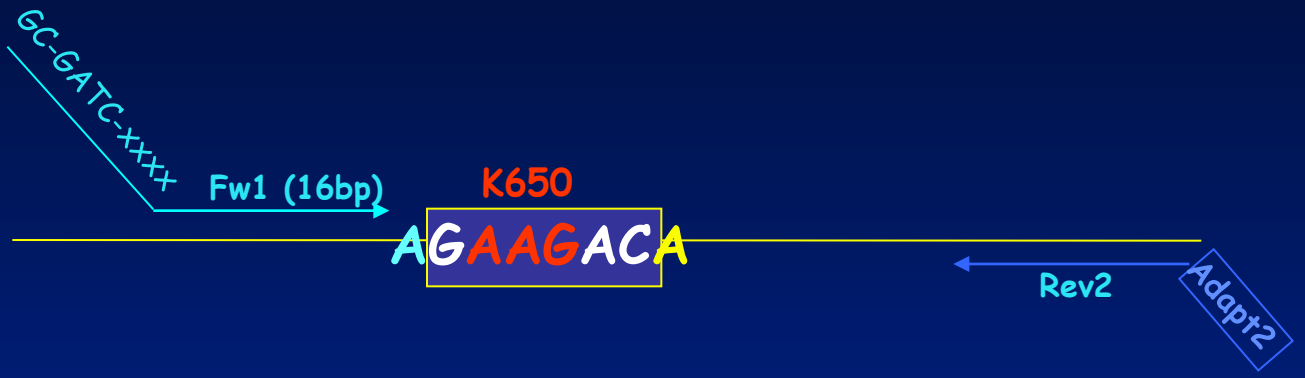
Acanthosis Nigricans (AN)
(Berk *et al Arch Dermatol* 07)

# 2007: Quantifying *FGFR3* K650 mutations in sperm?

Genomic DNA ± Spike DNA (HCH1)

*BbsI* digestion



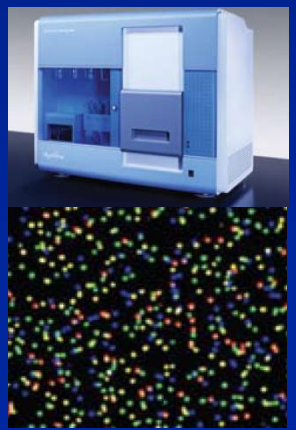




PCR amplification of *BbsI*-resistant fragment

*BbsI* digestion

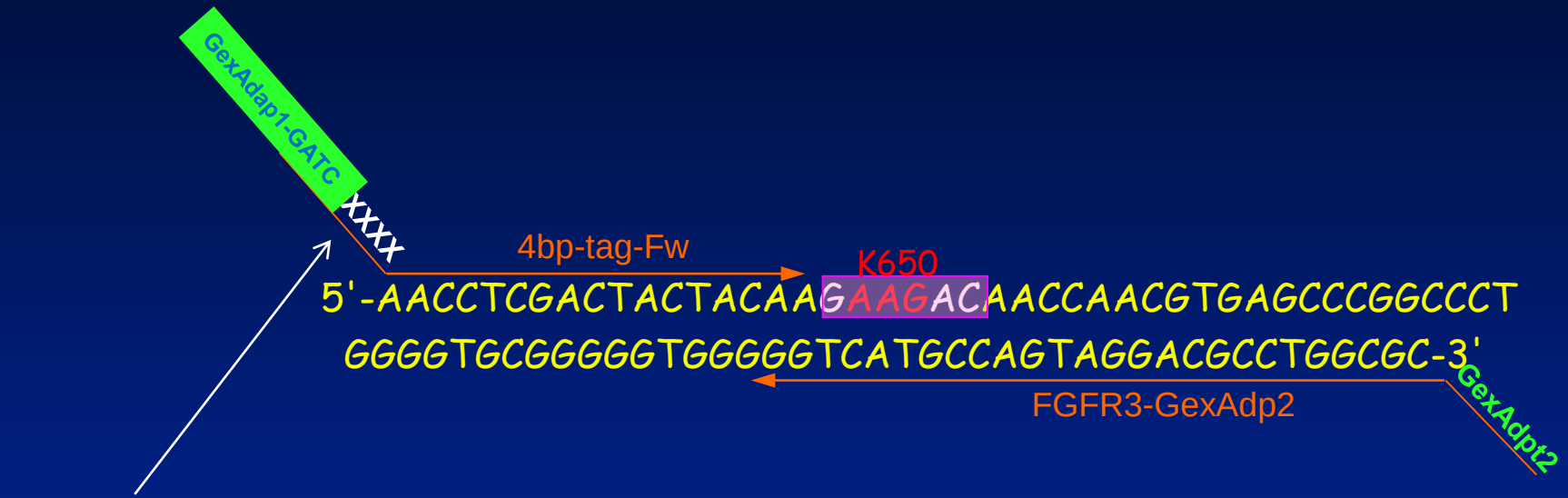
2nd PCR with nested primer containing unique 4bp ID tags

Illumina sequencing



Goriely *et al*, *Nat Genet*, 2009

# Construction of the GAII libraries for unidirectional sequencing with Gex-*Dpn*II primer



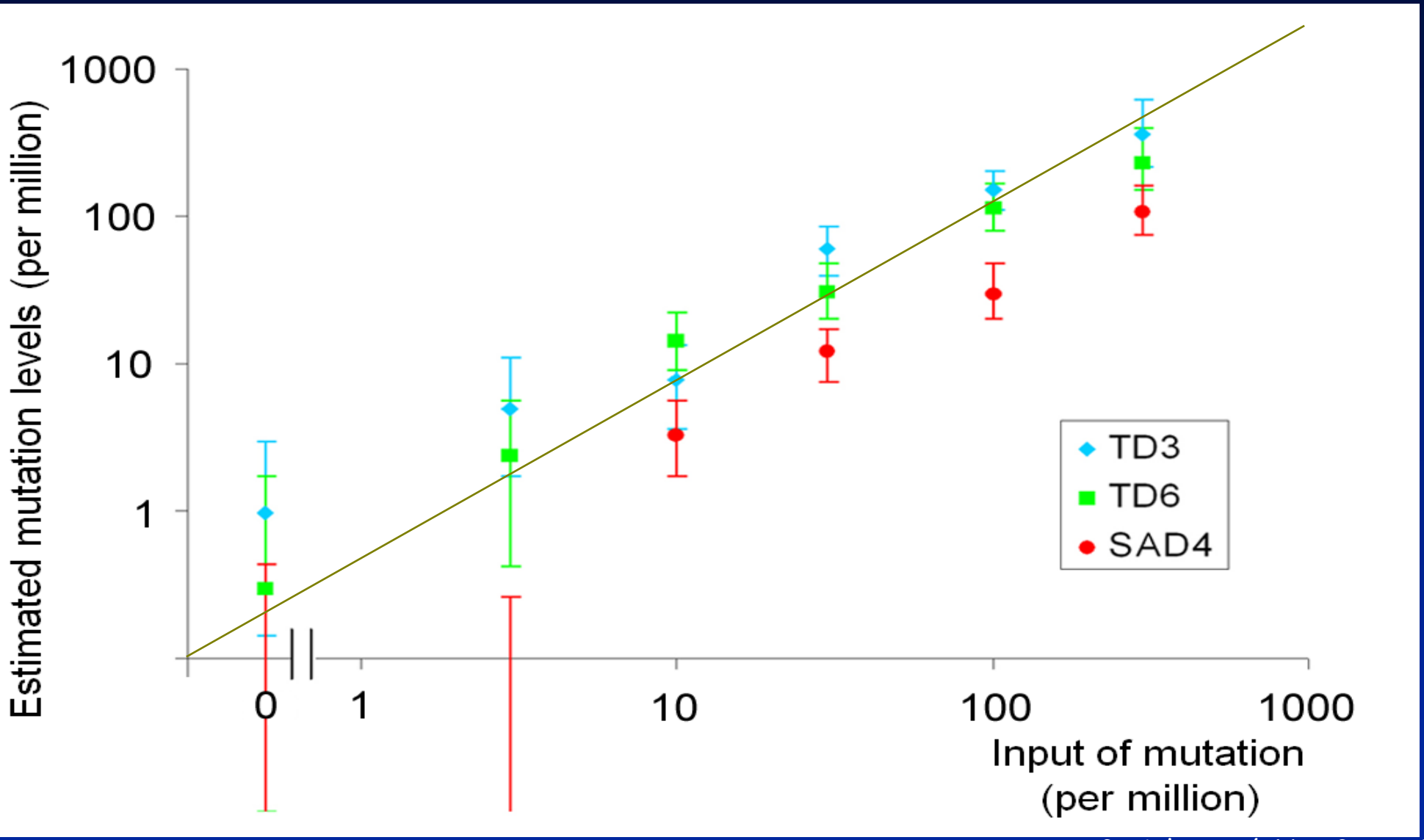

112 unique XXXX Tags

All 112 samples mixed in equimolar ratio

## Sequencing scheme


Goriely *et al*, *Nat Genet*, 2009

# Illumina sequencing accurately quantifies mutation levels




Goriely *et al*, *Nat Genet*, 2009

# 3 lanes of 36-bp unidirectional GAII sequencing

| No spike | Spike @ 1:100,000 | Spike @ 1:10,000 |
|---|---|---|
| ACGT--- | ACGT--- | ACGT--- |
| AGTC--- | AGTC--- | AGTC--- |
| GATT--- | GATT--- | GATT--- |
| TGCA--- | TGCA--- | TGCA--- |
| TGCA--- | TGCA--- | TGCA--- |
| GTTC--- | GTTC--- | GTTC--- |
| TGAC--- | TGAC--- | TGAC--- |
| GGTA--- | GGTA--- | GGTA--- |
| ACCT--- | ACCT--- | ACCT--- |
| GAAT--- | GAAT--- | GAAT--- |
| AACT--- | AACT--- | AACT--- |
| GACC--- | GACC--- | GACC--- |
| GGTT--- | GGTT--- | GGTT--- |
| TTGT--- | TTGT--- | TTGT--- |
| AATG--- | AATG--- | AATG--- |
| ACCT--- | ACCT--- | ACCT--- |
| AGCT--- | AGCT--- | AGCT--- |
| AGGC--- | AGGC--- | AGGC--- |
| AGCC--- | AGCC--- | AGCC--- |
| ..... 112X | ..... 112X | ..... 112X |

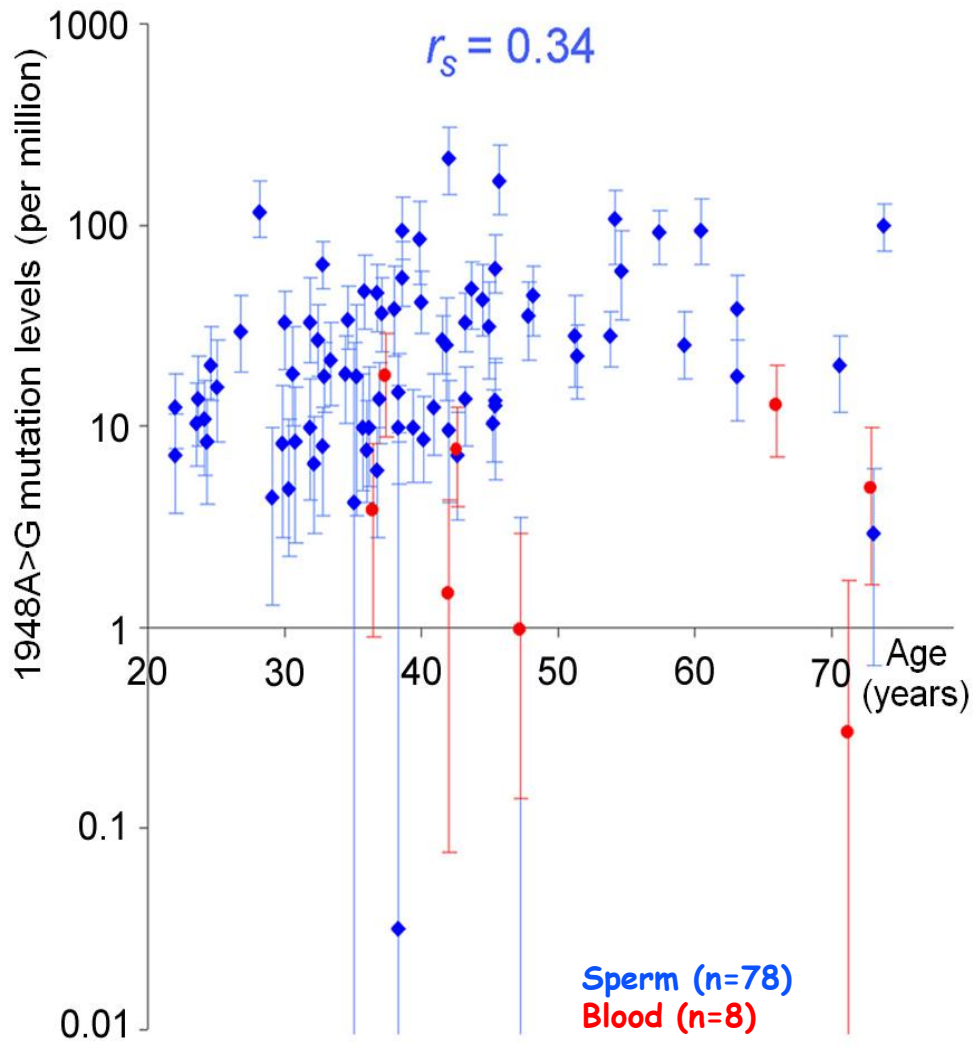
3 x 10µg of starting DNA for each sperm sample analysed ( = 10 million DNA copies)

After filtering, total of $3.10^6$ – $6.10^6$ tagged sequences per lane → each lane provides ~40,000 (30,000-60,0000) reads per sample

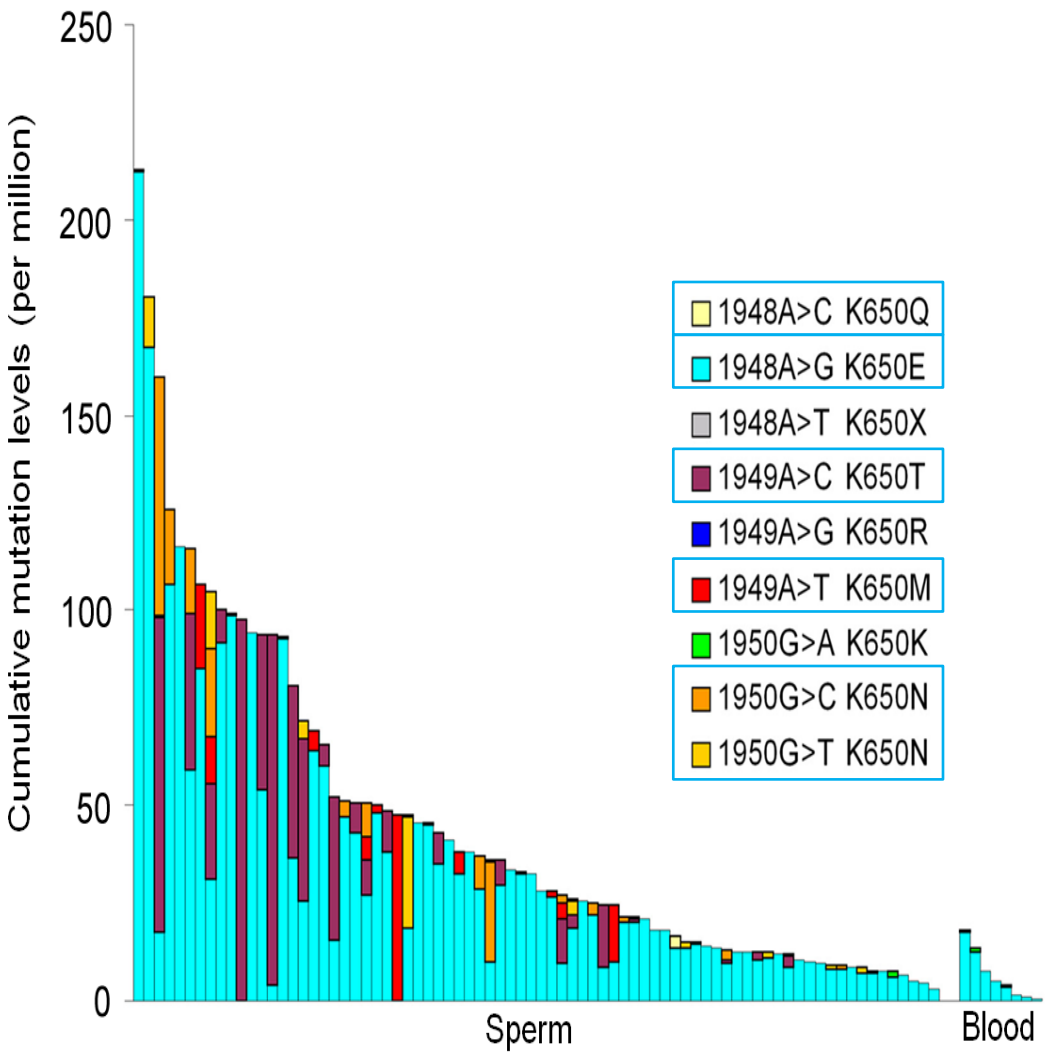
Allowing a large dynamic range of mutation quantification <1:1,000,000 – 1:100

Goriely *et al*, *Nat Genet*, 2009

# Quantification of K650 FGFR3 mutations




Levels of TDII mutation



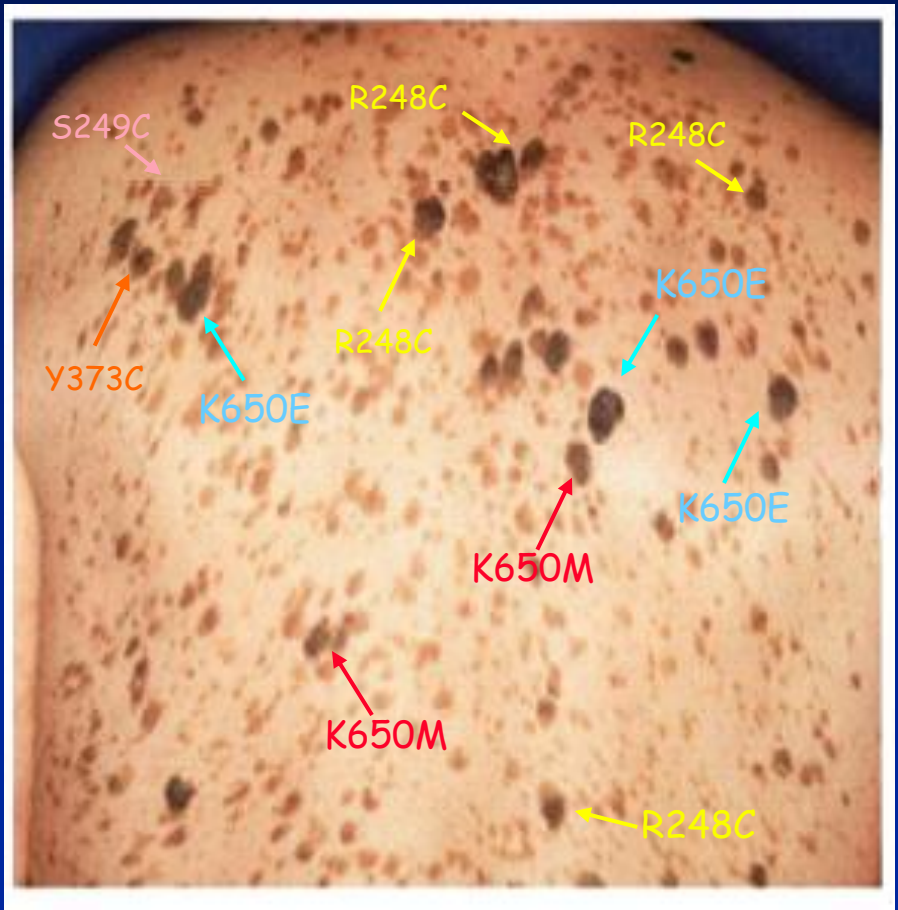

Cumulative levels of all K650 codon mutations

Goriely *et al*, *Nat. Genet.*, 2009

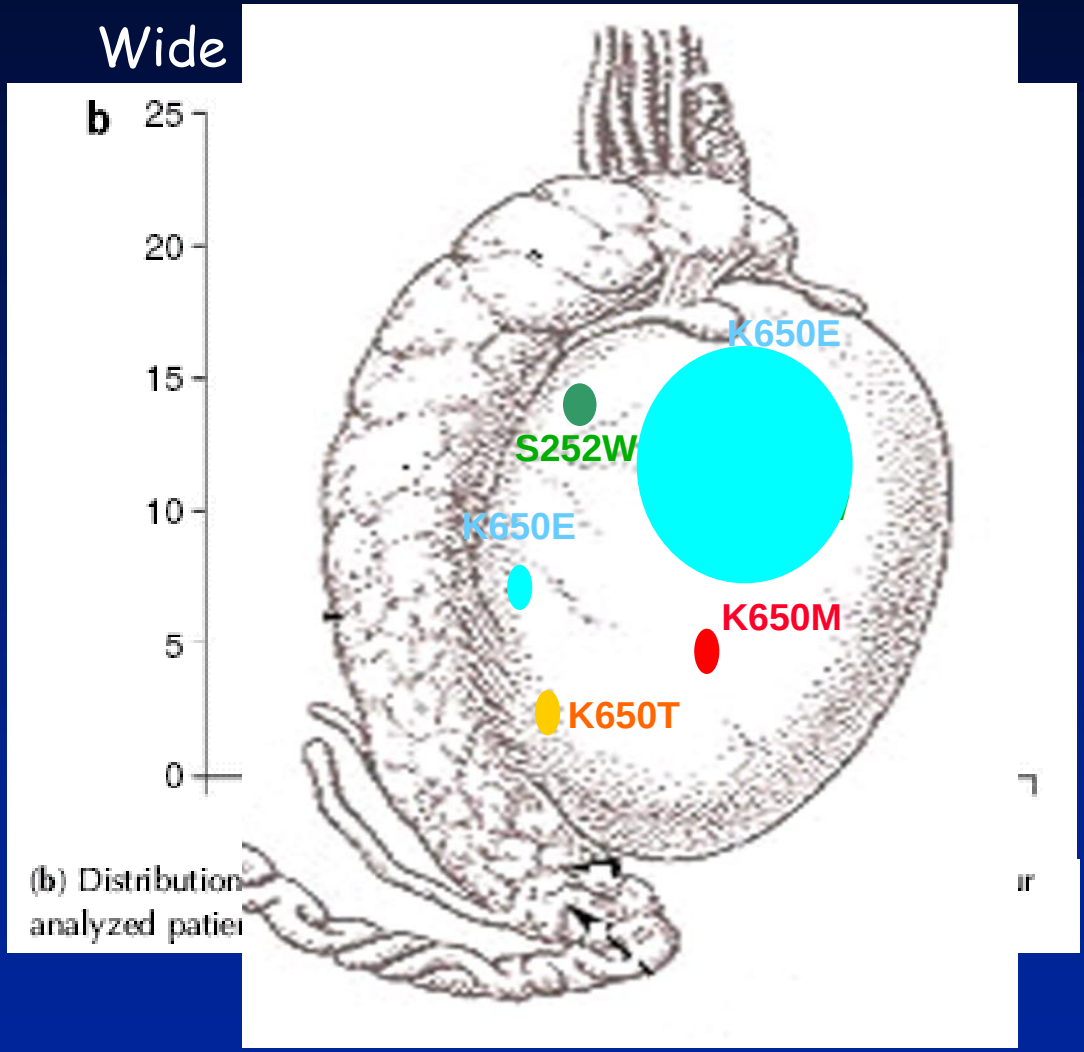
# TD mutations in benign skin tumours

## Seborrheic Keratoses

80-100% of people over 50y
Average 70 moles in people >75y




Wide

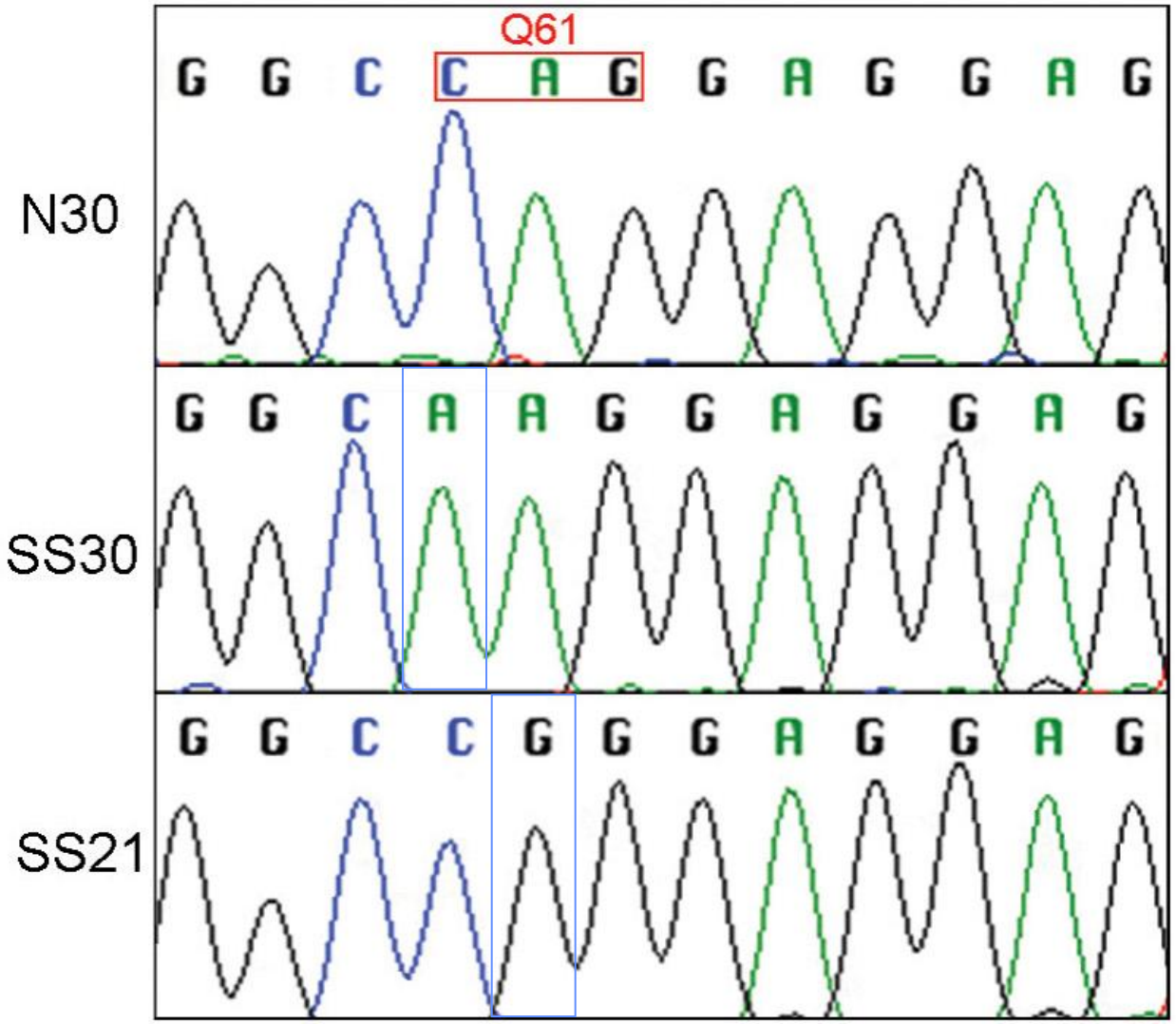



These 'selfish mutations' produce 'moles' in the testis that can develop into tumours

# *HRAS* Q61 mutations in testicular tumours

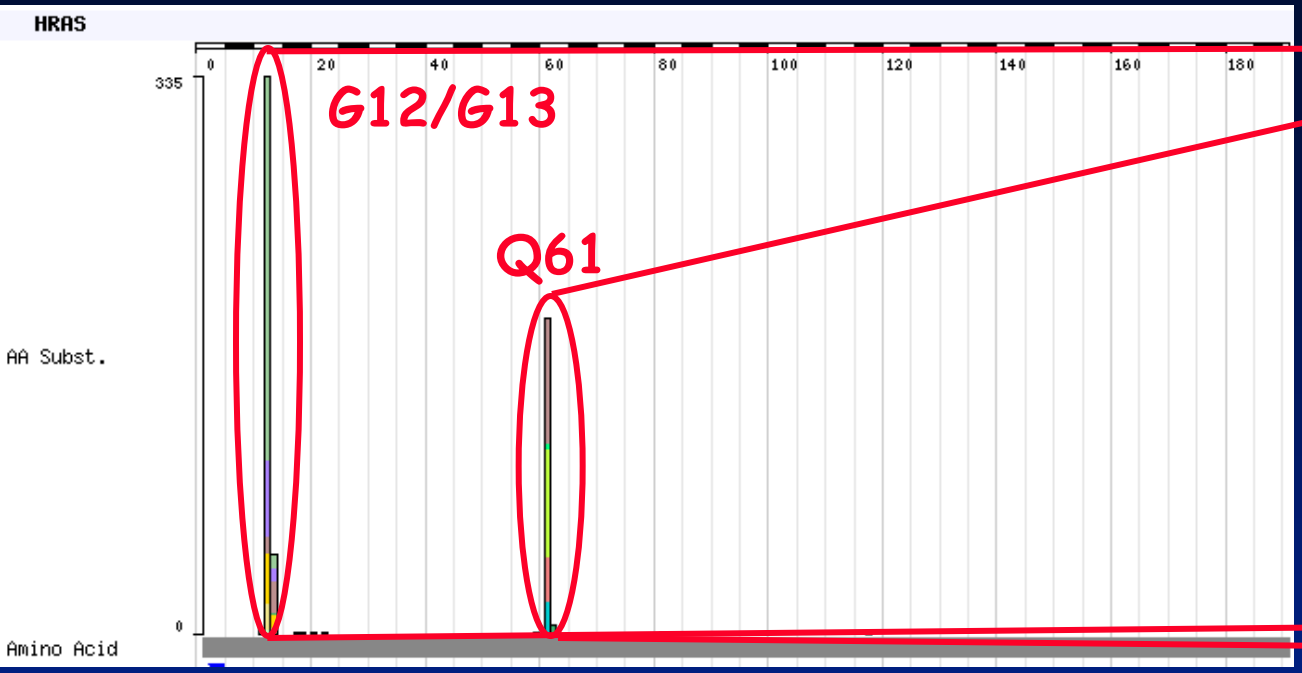
2 homozygous 181 C>A
Q61K

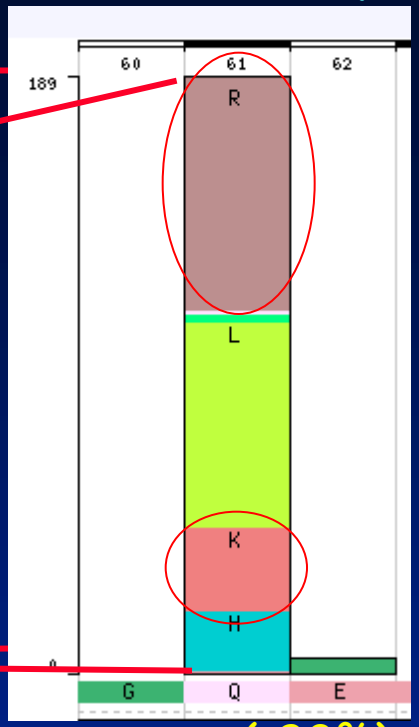
3 homozygous 182A>G
Q61R




Goriely *et al*, *Nat Genet*, 2009

# *HRAS* mutations in cancer and Costello syndrome




G12S is the most common (>90%)

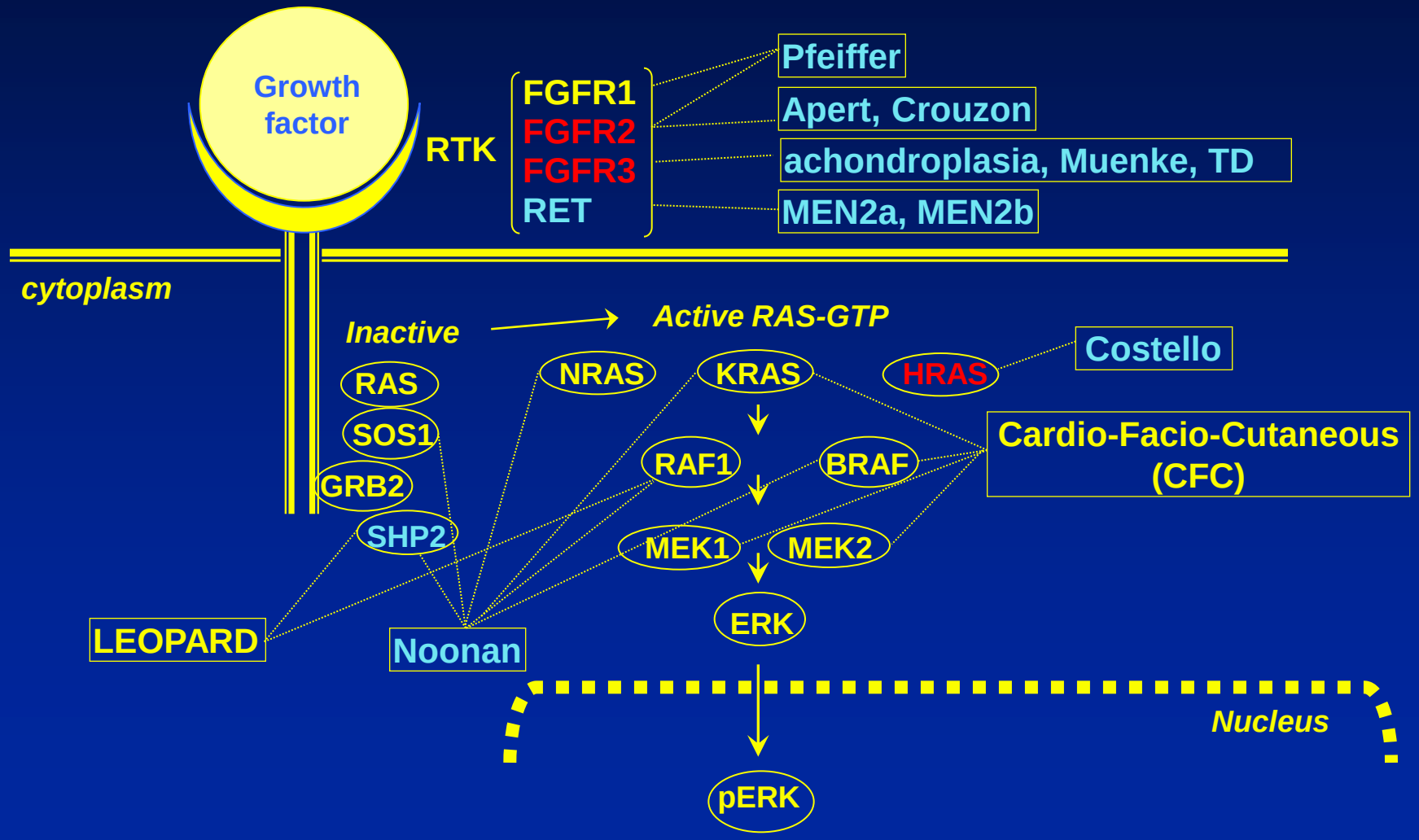
Kutsche *et al*, *Clin Genet* (2007)

COSMIC

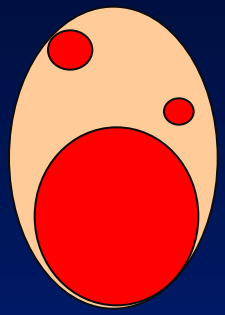
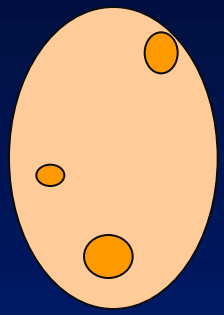
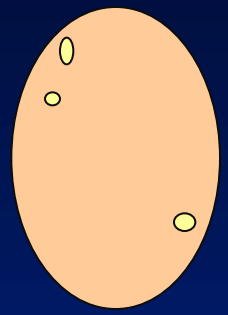
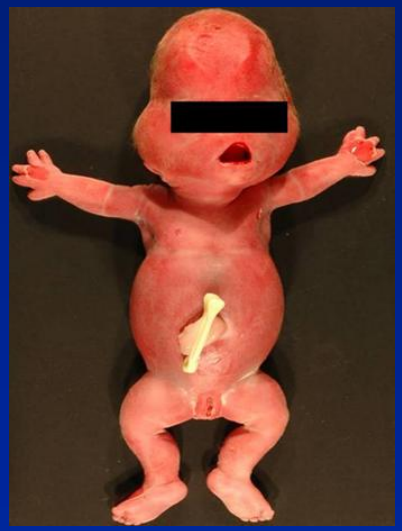
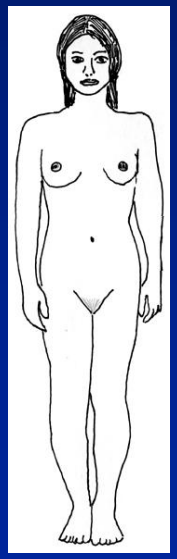
Are *HRAS* Q61 mutations lethal?

COSMIC, *www.sanger.ac.uk/genetics/CGP/**cosmic/***

# All paternal age-effect genes encode components of growth factor receptor-RAS-MAPK signalling pathway




Goriely *et al., Nat Genet*, 2009

| | Strongly activating mutation<br>e.g. FGFR3 K650E | Moderately activating mutation<br>e.g. FGFR3 G380R, FGFR2 S252W or HRAS G12S | Weakly activating mutation<br>e.g. rare sequence variants? |
|---|---|---|---|
| Effect of the *de novo* mutation in the testis (clonal expansion) |  |  |  |
| Consequence as a somatic mutation in the testis | Sperm enrichment 100-1000x<br>Spermatocytic seminoma | Sperm enrichment ~ 100x<br>Clones in the testis | Sperm enrichment >1-50x |
| Consequence as a germline mutation in the embryo |  <br>Lethal disorder |  <br>Classical paternal age effect disorders |  <br>Disease predisposition? |

Goriely *et al., Nat Genet*, 2009

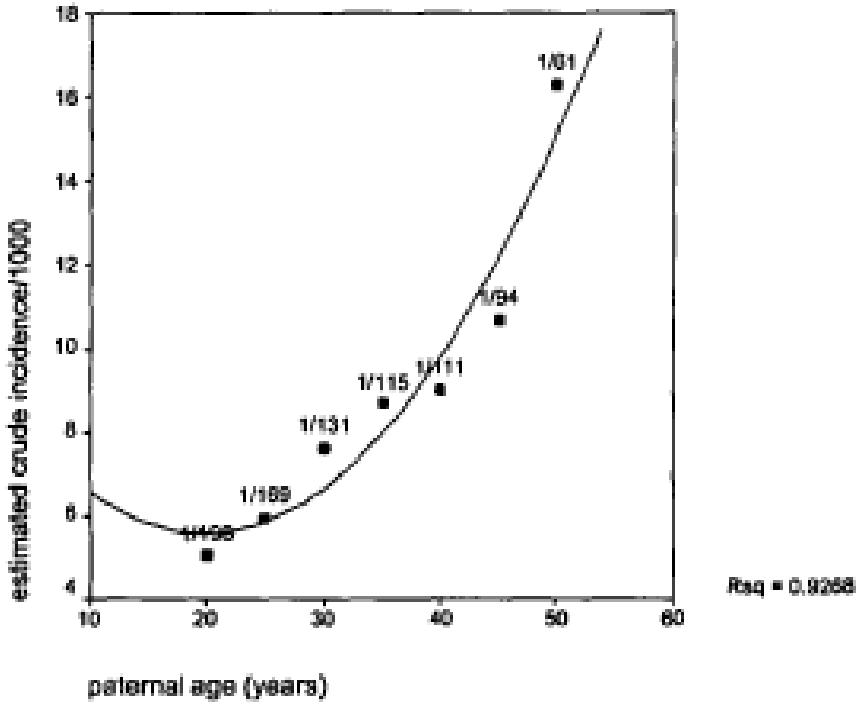
# Autism and parental age

## Paternal Factors and Schizophrenia Risk: De Novo Mutations and Imprinting

*Schizophrenia Bulletin*, Vol. 27, No. 3, 2001

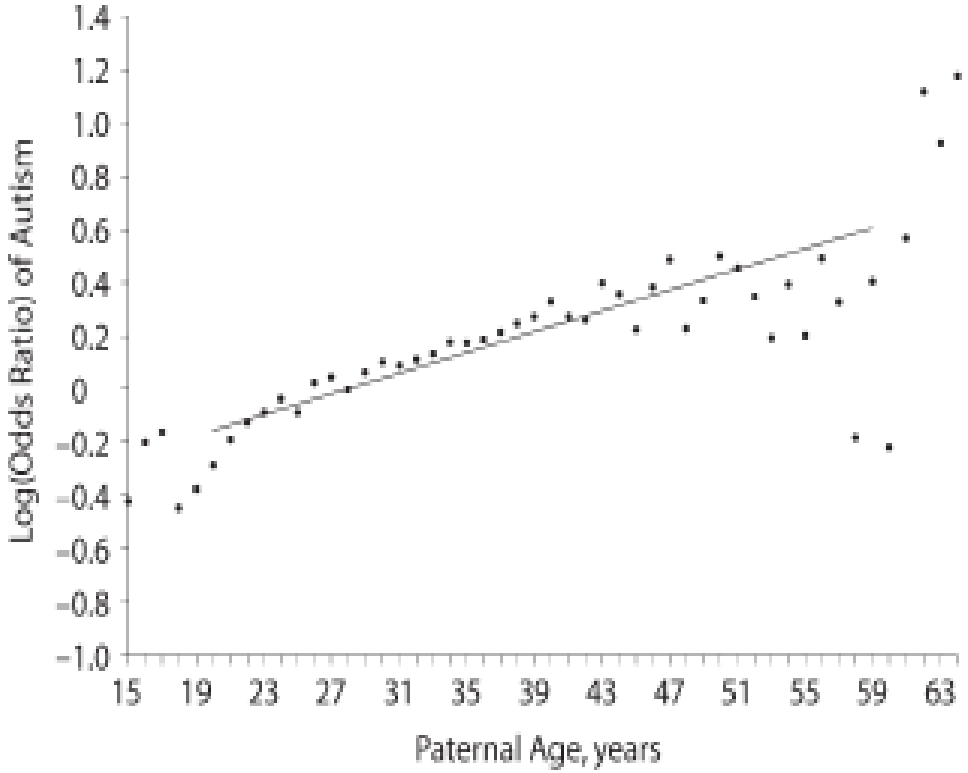
by *Dolores Malaspina*

**Figure 1. An estimated relationship between paternal age and schizophrenia risk**




Grether et al, 2009

*Am J Epidemiol* 2009;170:1118–1126




Does selfish PAE selection process contribute to the burden of mutations in complex disorders?

# Are PAE mutations the tip of the iceberg of a more common mechanism generating genetic heterogeneity?

How many 'weakly' pathogenic mutations are selected in the ageing testis?

## Analysis of Genetic Inheritance in a Family Quartet by Whole-Genome Sequencing

Jared C. Roach,[1*] Gustavo Glusman,[1*] Arian F. A. Smit,[1*] Chad D. Huff,[1,2*] Robert Hubley,[1] Paul T. Shannon,[1] Lee Rowen,[1] Krishna P. Pant,[3] Nathan Goodman,[1] Michael Bamshad,[4] Jay Shendure,[5] Radoje Drmanac,[3] Lynn B. Jorde,[2] Leroy Hood,[1†] David J. Galas[1†]

*Science* 30 April 2010: Vol. 328. no. 5978, pp. 636 - 639

Intergenerational spontaneous mutation rate = ~ 1.1 $x10^{-8}$
~70 new mutations/diploid genome

Paternal >> maternal contribution?
Recurrent hits in PAE genes?
Recurrent hits in genes of a given pathway such as GF-RAS-MAPK?

# Other applications of multiplex very high coverage Illumina reads?

- 'Personalised Medicine': Doesn't necessarily require a WGS!!

  - Decide on best treatment depending on tumour mutational profile (Kras mutational status in colorectal cancer (for EGFR antibodies treatment))

  - Monitoring emergence of resistant mutations (such as EGFR T790M in lung cancer (and use of 2nd generation TKI)

- Non-invasive diagnostic:

  - Prenatal diagnostic on free foetal DNA (5-10%)

  - Routine detection of colorectal neoplasia from stools, blood or urine in high-average risk populations

  - Molecular detection of pre-cancerous lesions in NSCLC lung cancer (EGFR L858R (or Deletion ex 19) ( =20,000 cases in US/year)

  - Monitoring residual disease and tumour recurrence after chemotherapy treatment

# Thanks to…


WIMM (Oxford)
Ruth Hansen
Indira Taylor
Simon McGowan (CBRG)

Statistics Dept (Oxford)
Gil McVean
Susanne Pfeifer

Andrew Wilkie

Pyrosequencing (Uppsala, Sweden)
Bjorn Ingemarsson
Maria Rojmyr

Copenhagen University Hospital
Ewa Rajpert-DeMeyts
Grete Krag Jacobsen

Oxford Fertility Clinic
Anonymous sperm donors

wellcome trust