Sequencing of neuroblastoma From Mutations to Phenotypes

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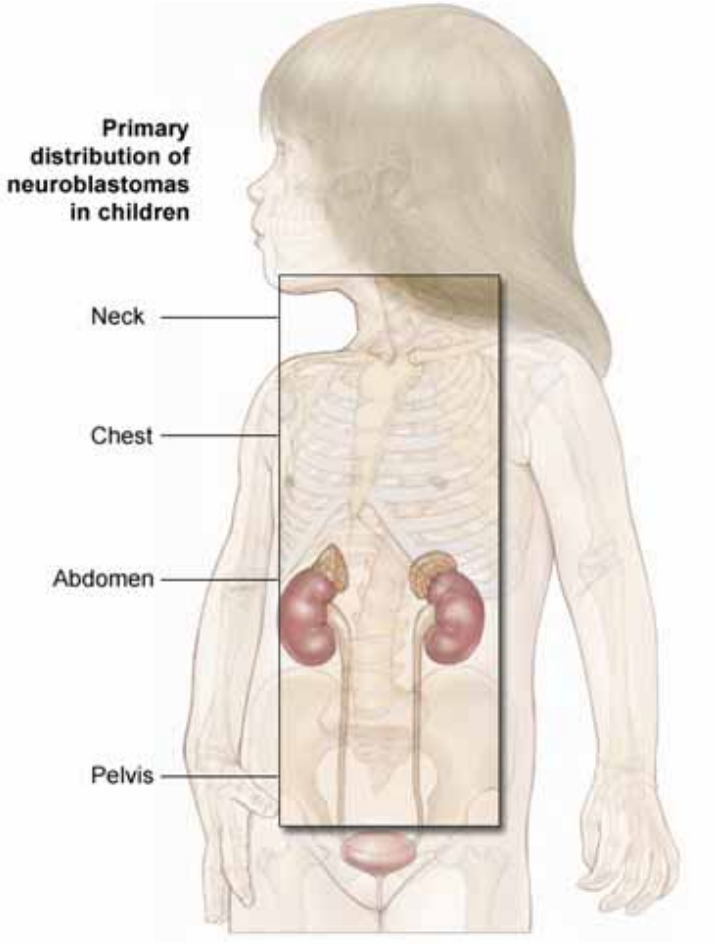
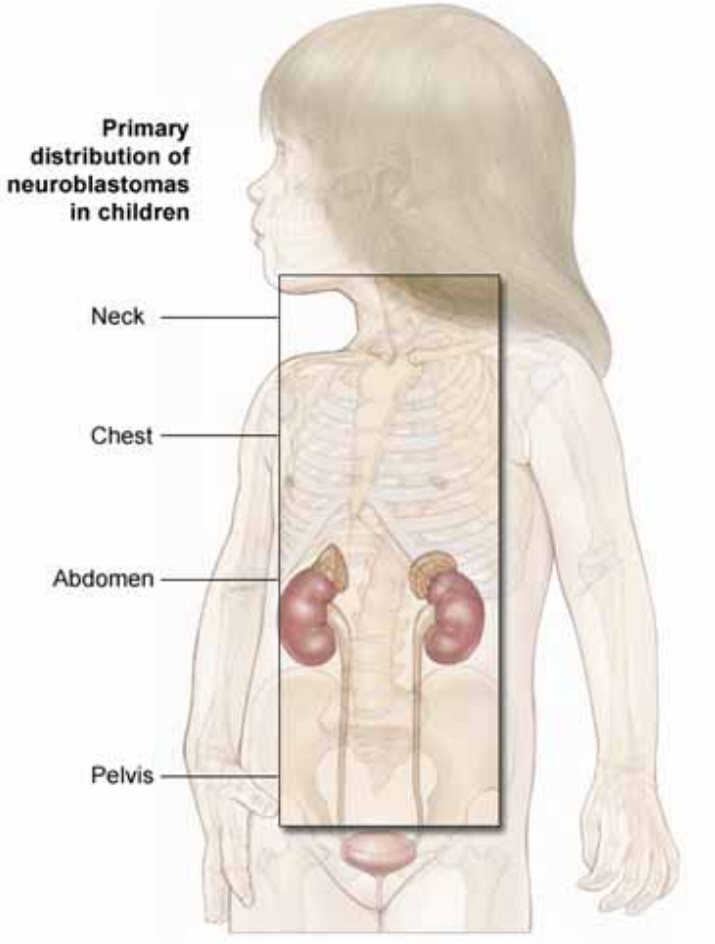
Neuroblastoma

- Neuroblastoma
  - Childhood neuro endocrine tumor
  - Peripheral nervous system
    - Neural crest derived
    - Neuroblast should normally form neurons
  - Young children (mostly 0-3 years)



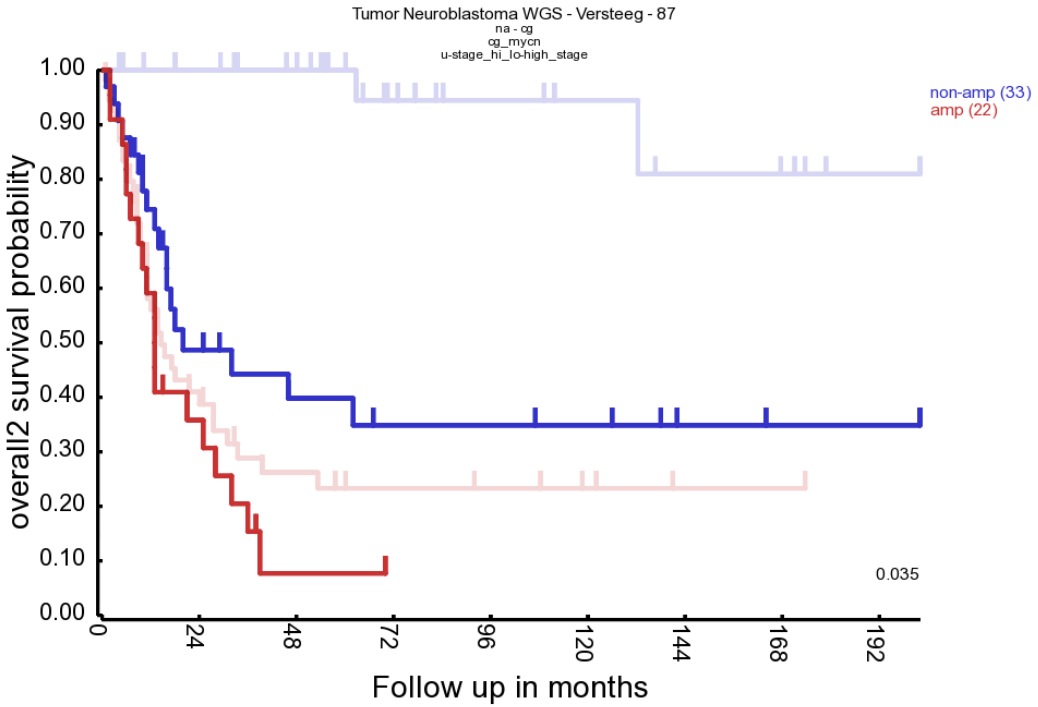
Neuroblastoma

- Pathogenesis of neuroblastoma
  - Variable clinical outcome
    - Low INSS stages (1,2)
      - Good prognosis
      - Numeric changes of chromosomal copy numbers
    - High INSS stages (3,4)
      - Poor prognosis
      - Structural chromosomal defects (LOH1p / 11q etc)
    - Special stage (4S)
      - Spontaneous regression



High stage neuroblastoma

- Poor prognosis
- Subgroup (~1/3) characterized by MYCN amplification
- Rest unknown

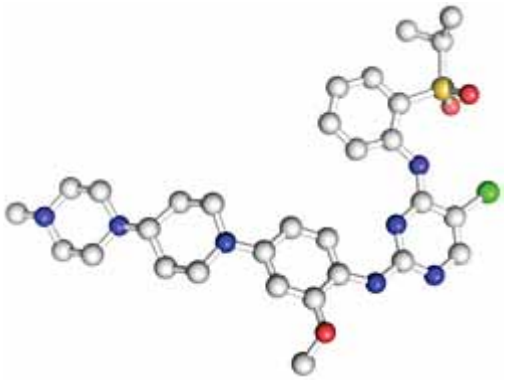


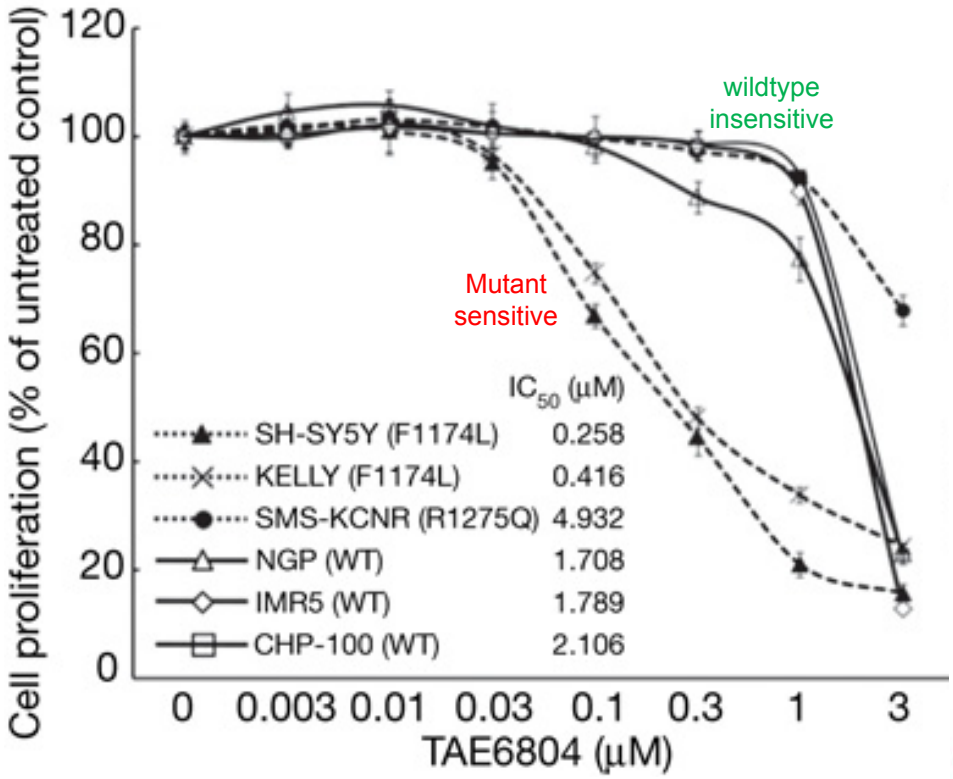
Gene defects in neuroblastoma

MYCN amplification	20%	(Schwab et al., 1983)
ALK	7%	(George/Mosse/Janoueix-Lerosey/Chen 2008)
Cyclin D1 amplification	4%	(Molenaar et al., 2003)
PHOX2B	4%	(van Limpt et al., 2004)
PTPRD	4%	(Stallings et al. 2006)
NF1	3%	(Hölzel et al., 2010)
PTPN11	2%	(Merks et al., 2004, Bentires-Alj et al., 2004)
FOXR1	1%	(Santo et al., 2011)
LIN28B	1%	(Molenaar et al., 2012)

Some defects can be targeted in therapy

- Gene defects can make tumors susceptible to intervention
  - Activated ALK (F1174L) can be inhibited by TAE-684





George et al, Nature, 2008

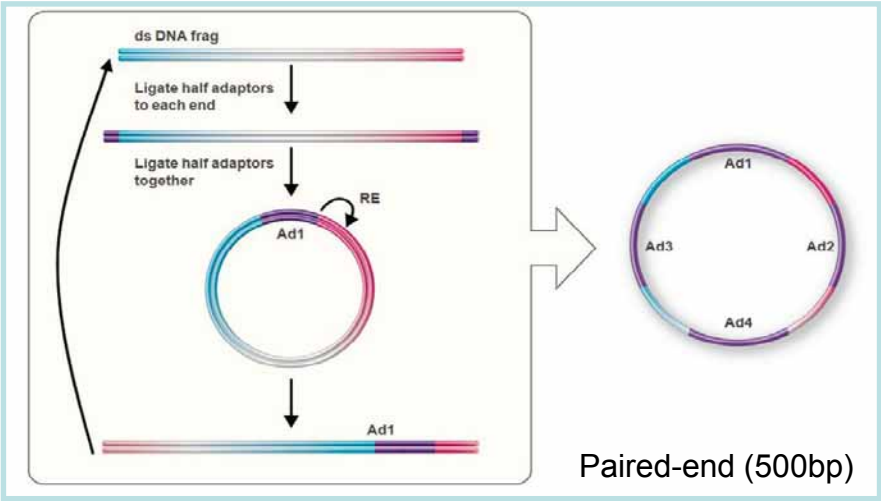
Which are the potential targets in NB?

- Assess the composition and copy number of every base in the entire genome
- Recent developments in sequencing technology make whole genome sequencing possible and affordable

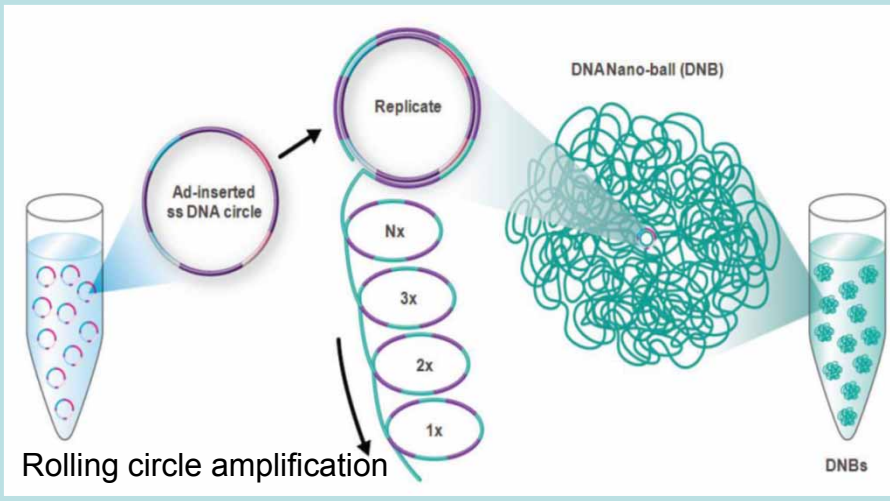
Complete genomics

amc Onco Genomics

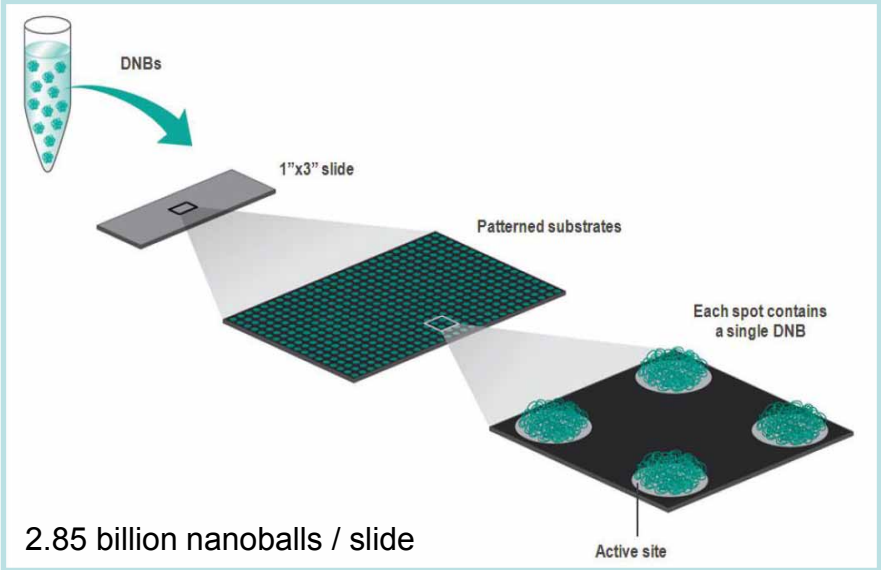
BGI/Complete Genomics WGS technology



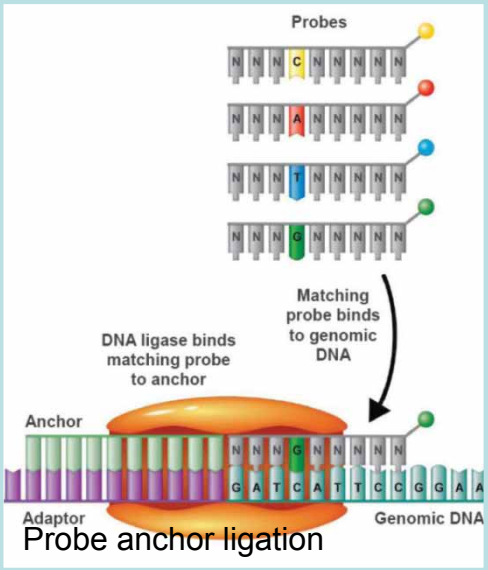
Paired-end (500bp)



Rolling circle amplification



2.85 billion nanoballs / slide

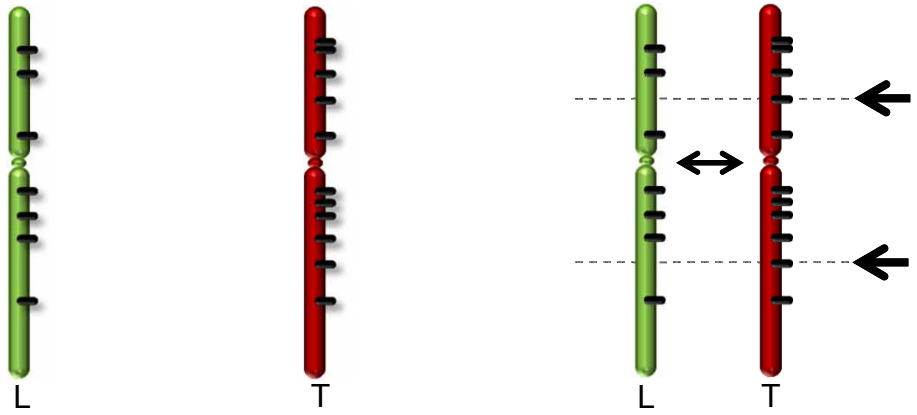


Probe anchor ligation

Advanced bioinformatics

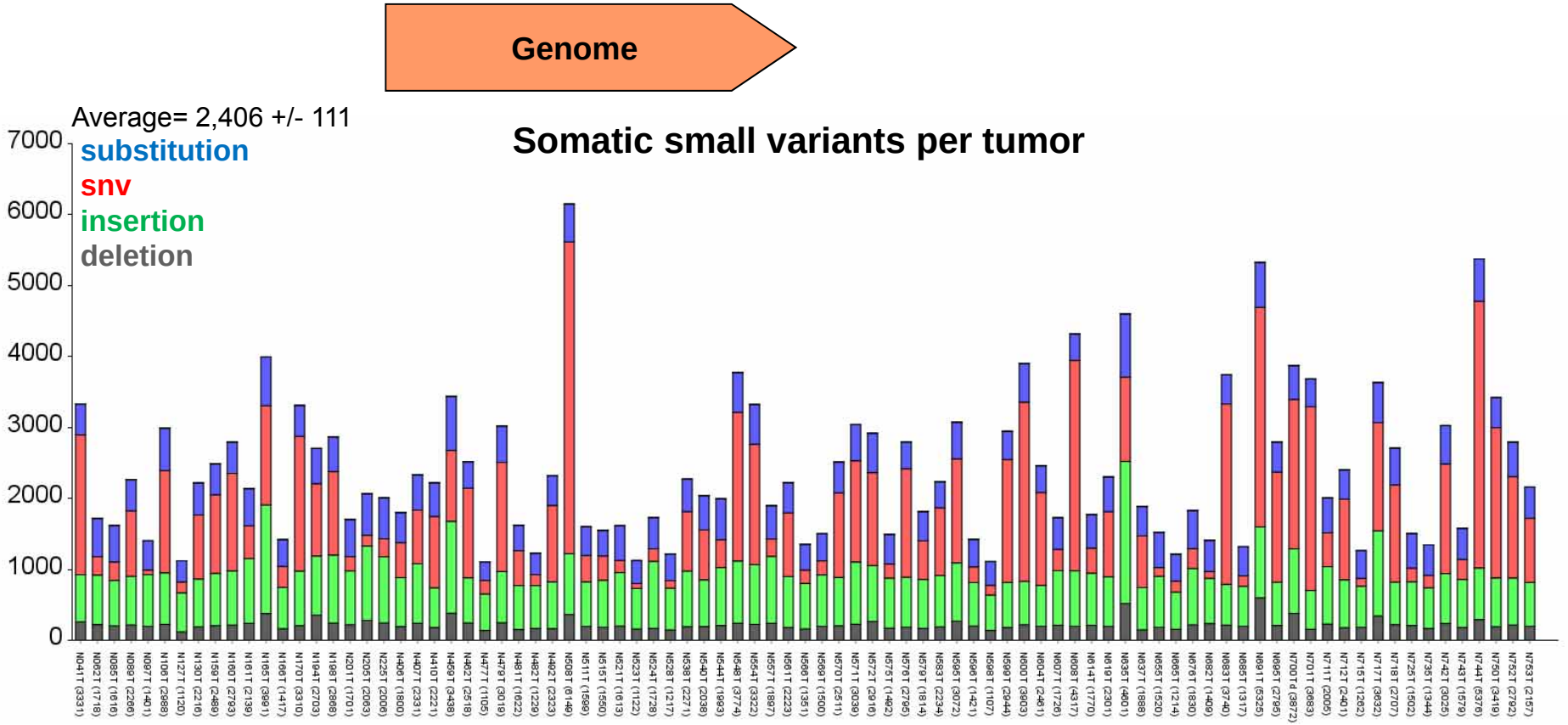
Somatic variants

- Compare tumor genome to normal genome
  - Subtract all events that are in common to both
  - Apply some quality filters



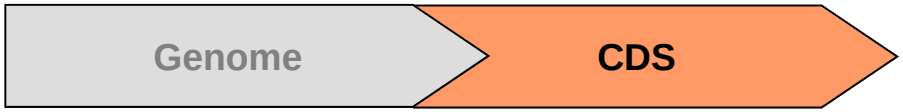
Differences with respect to Reference genome

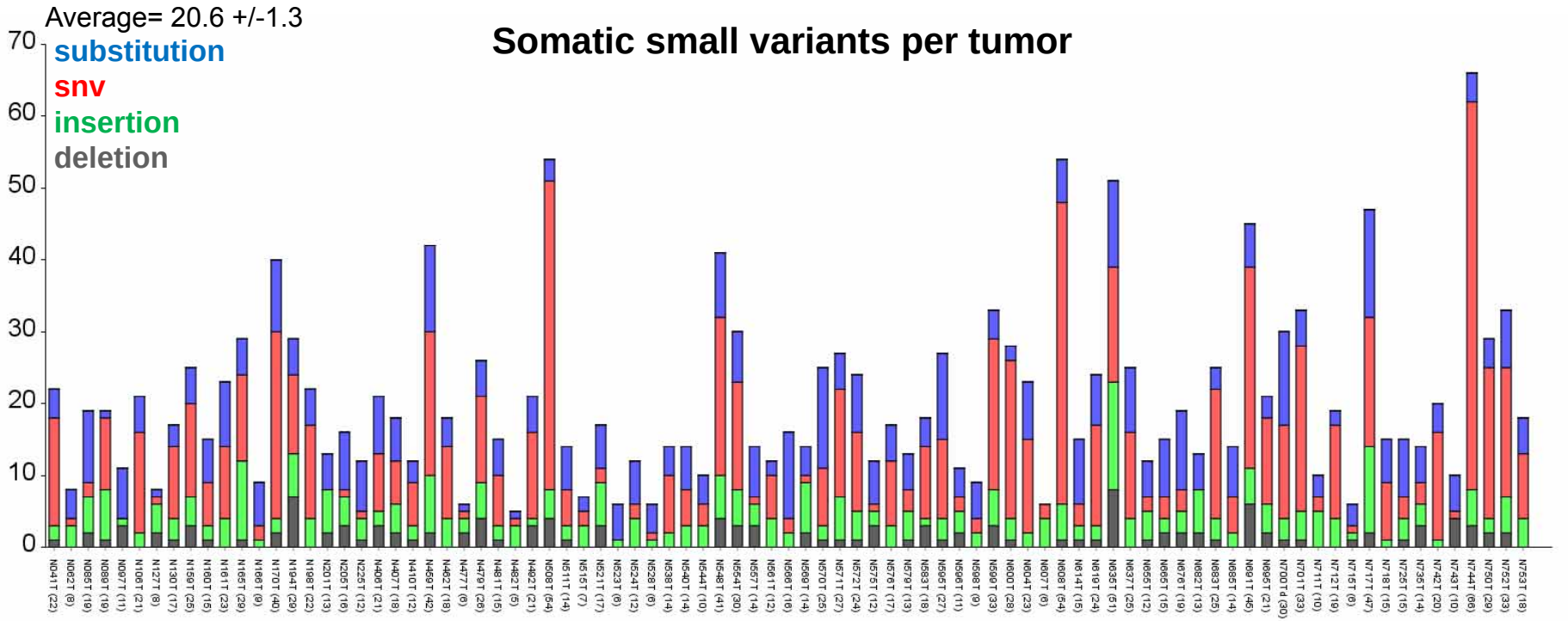
Somatic Tumor/Lymphocyte comparisons



Molenaar & Koster et al, Nature, 2012

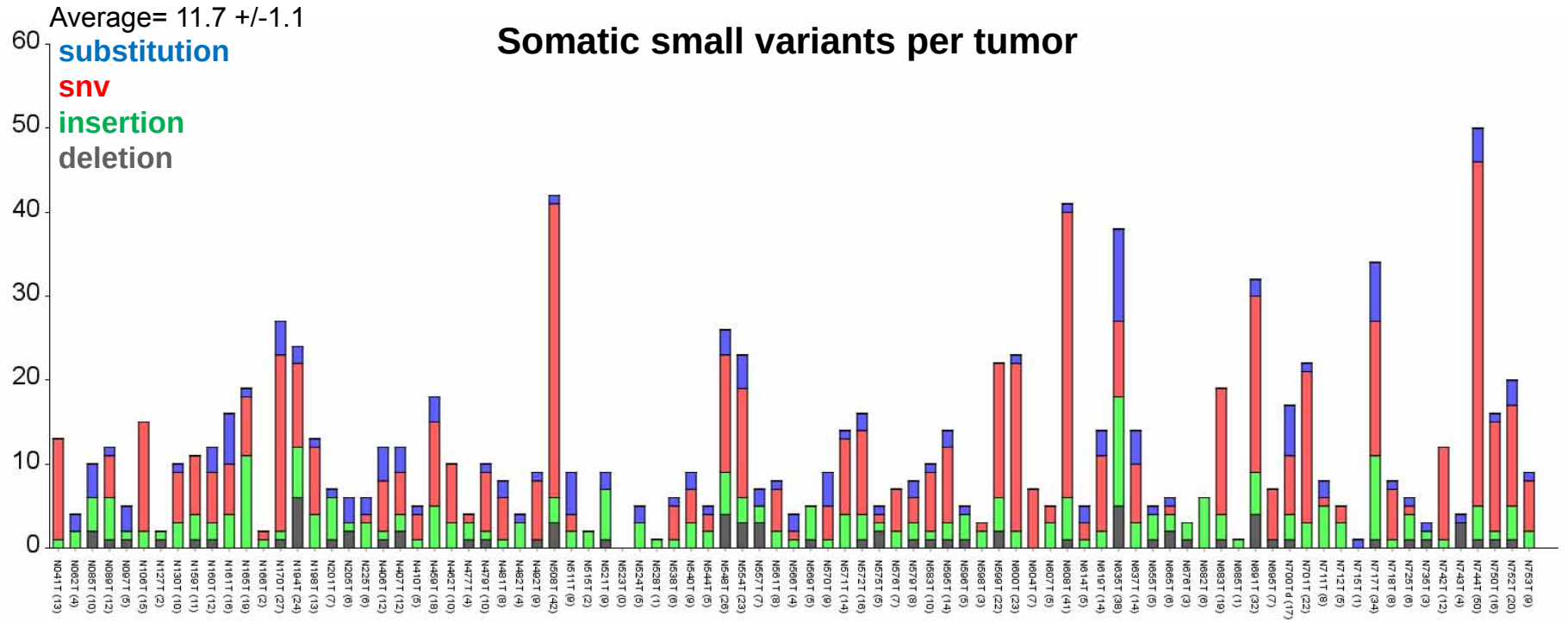
Somatic Tumor/Lymphocyte comparisons





Molenaar & Koster et al, Nature, 2012

Somatic Tumor/Lymphocyte comparisons



- **~ 12 mutations per patient**
- **Only 3 genes recurrent n>2 (ALK / FSIP2 / TIAM1)**
- **21 genes n=2**

Molenaar & Koster et al, Nature, 2012

Pediatric whole genome sequencing

- Paucity of somatic mutations is becoming a recurrent theme in pediatric cancers

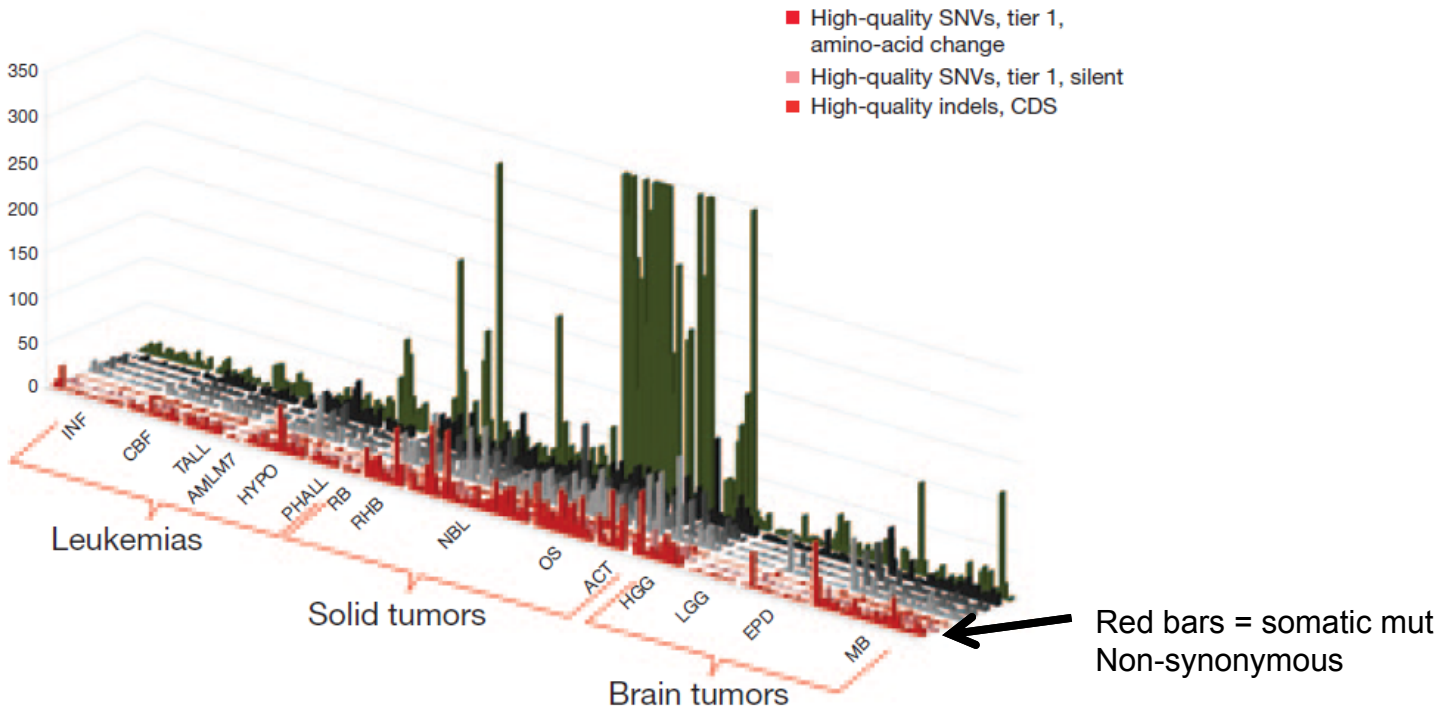


Figure 2 Genetic landscape of 15 different types of pediatric cancers determined from whole-genome sequencing of 260 tumors and matching germline samples.

Downing et at, Nat Genet, 2012

Pediatric whole genome sequencing

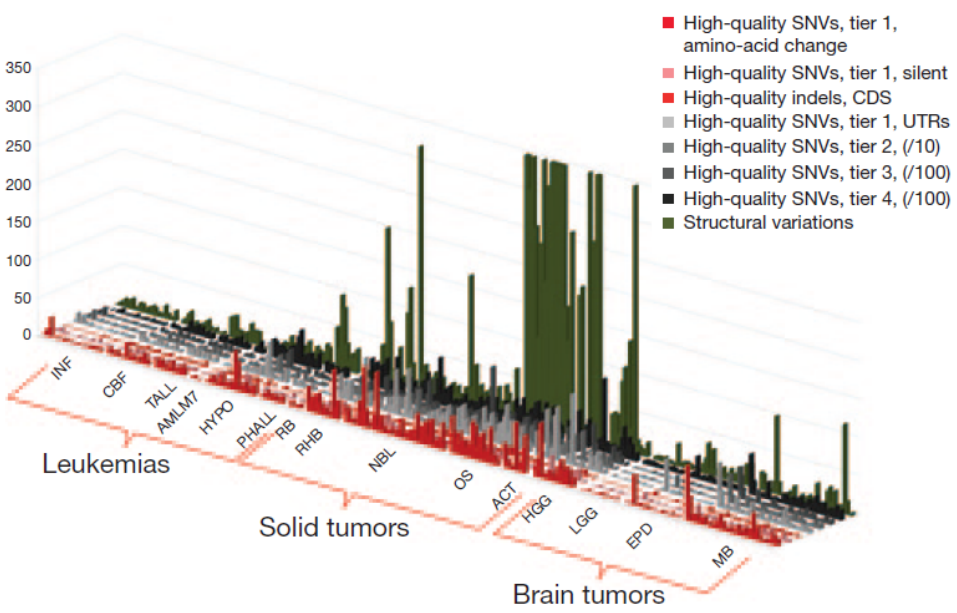
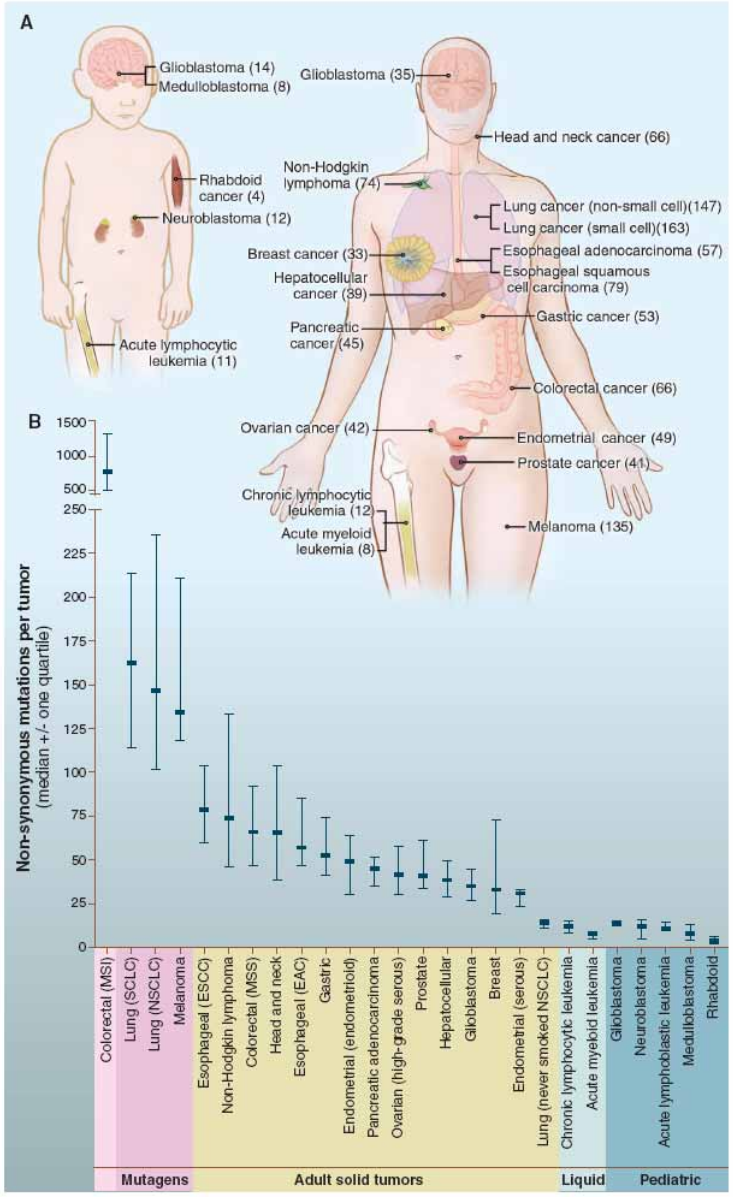


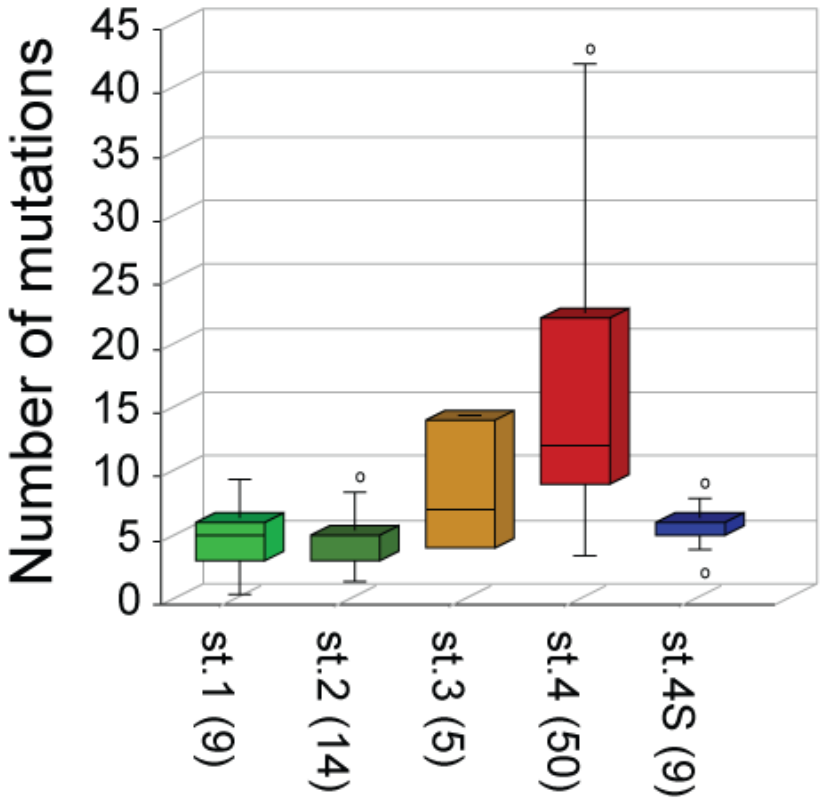
Figure 2 Genetic landscape of 15 different types of pediatric cancers determined from whole-genome sequencing of 260 tumors and matching germline samples. The number of somatic mutations in each sample,

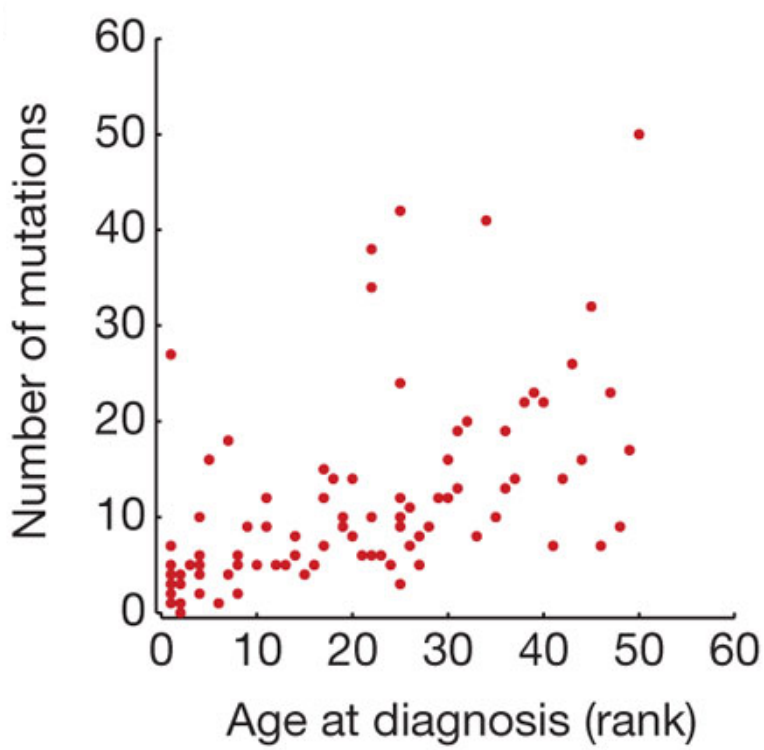
Downing et at, Nat Genet, 2012



Vogelstein et al, Science, 2013

Mutations correlate with stage and survival





Molenaar & Koster et al, Nature, 2012

Conclusions from WGS of neuroblastoma

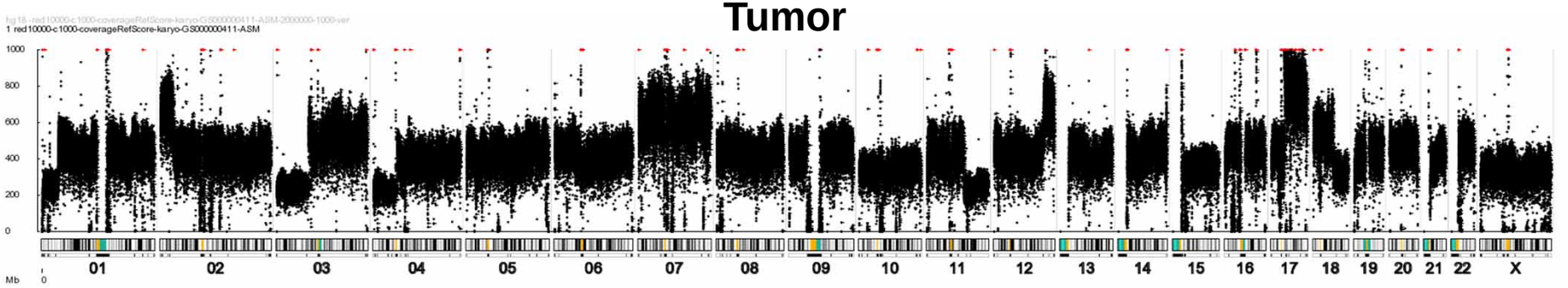
1 Low frequency of somatic mutations

Patient WGS Coverage

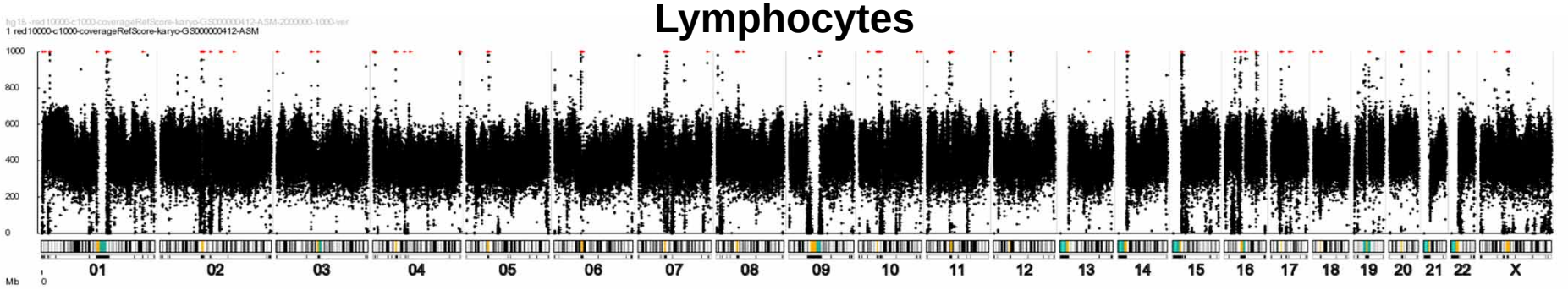
- On average, every base in a tumor, as well as its corresponding lymphocyte sample has been seen ~50 times (Coverage). This can be plotted on the genome

Patient WGS Coverage

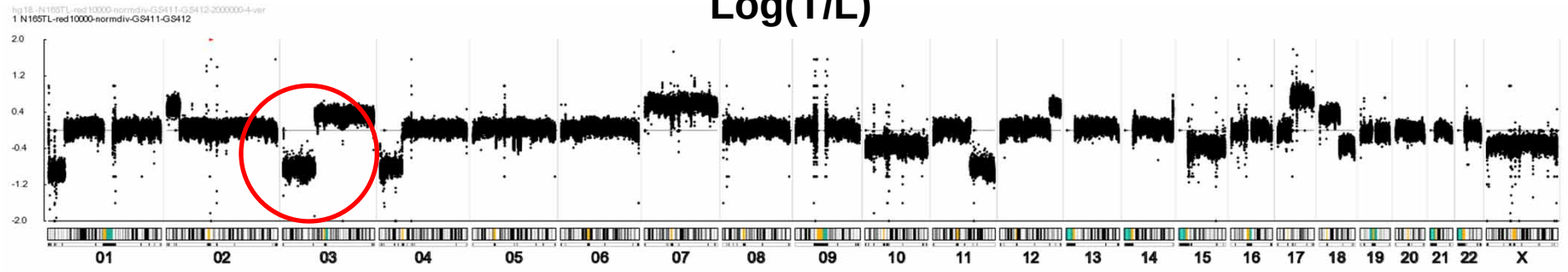
Tumor



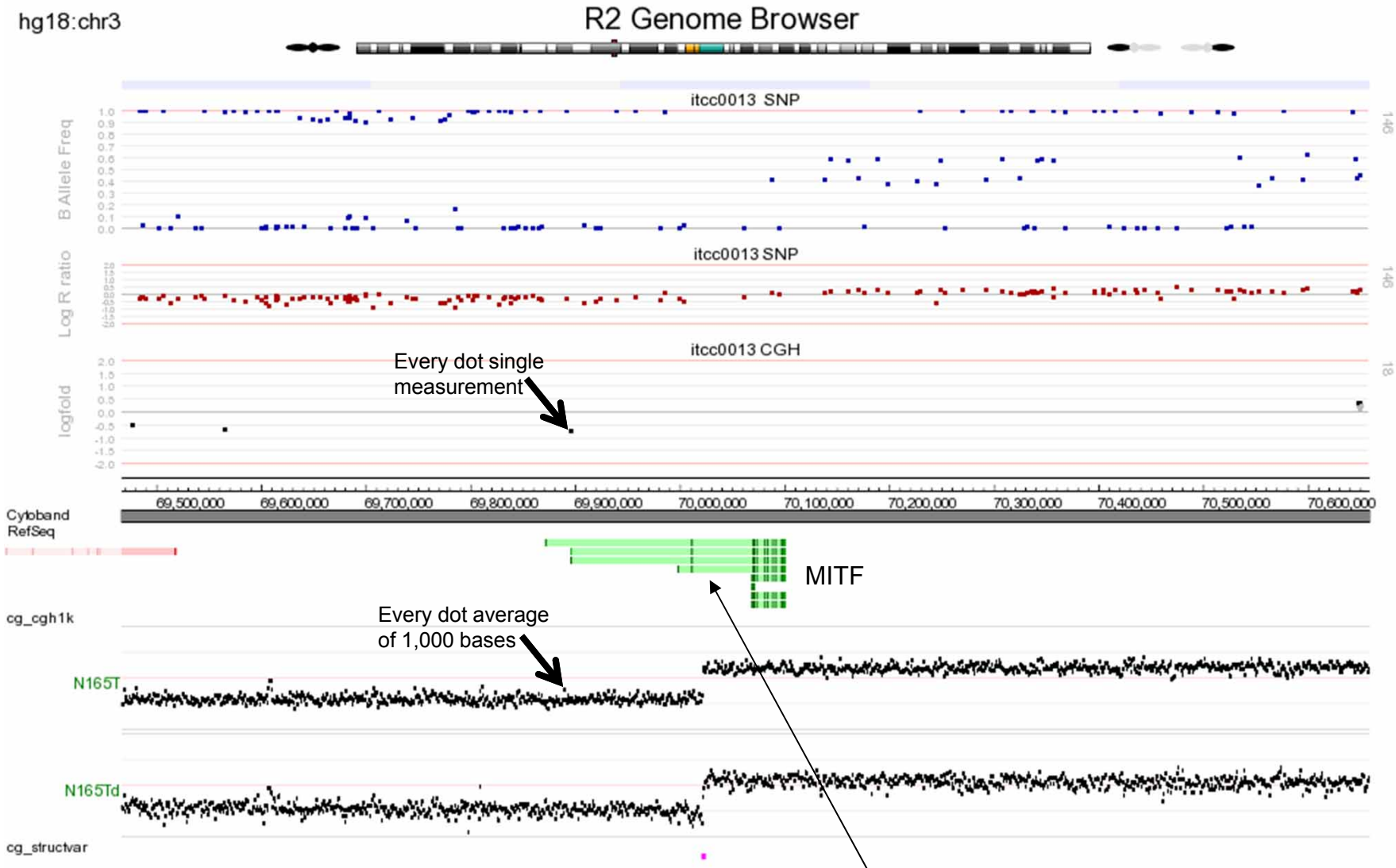
Lymphocytes



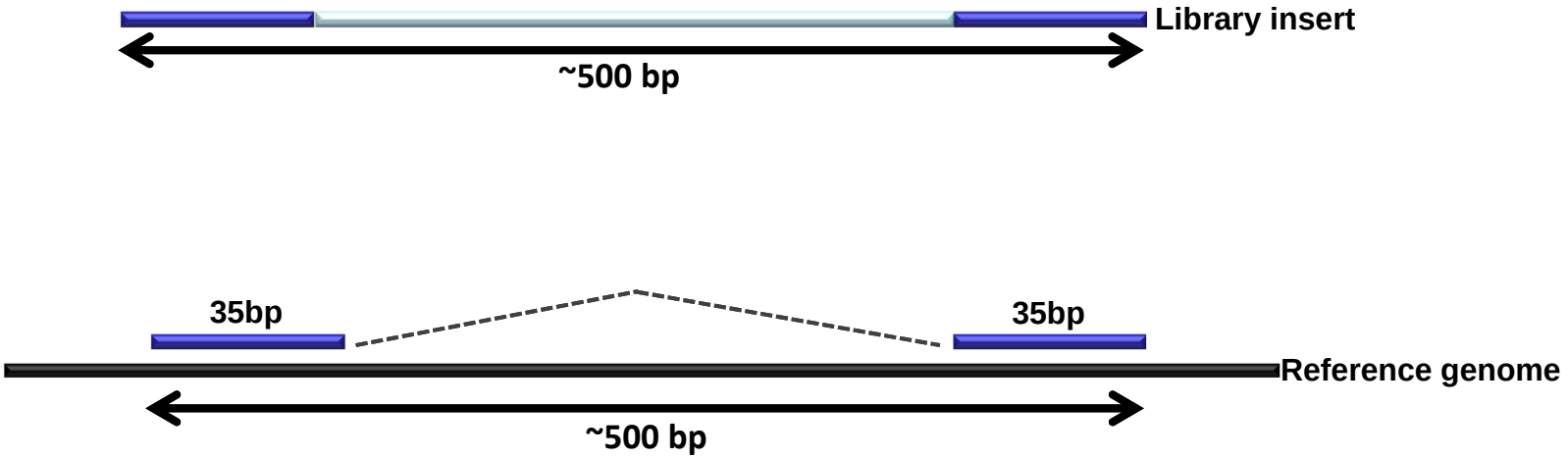
Log(T/L)



Ultra high resolution

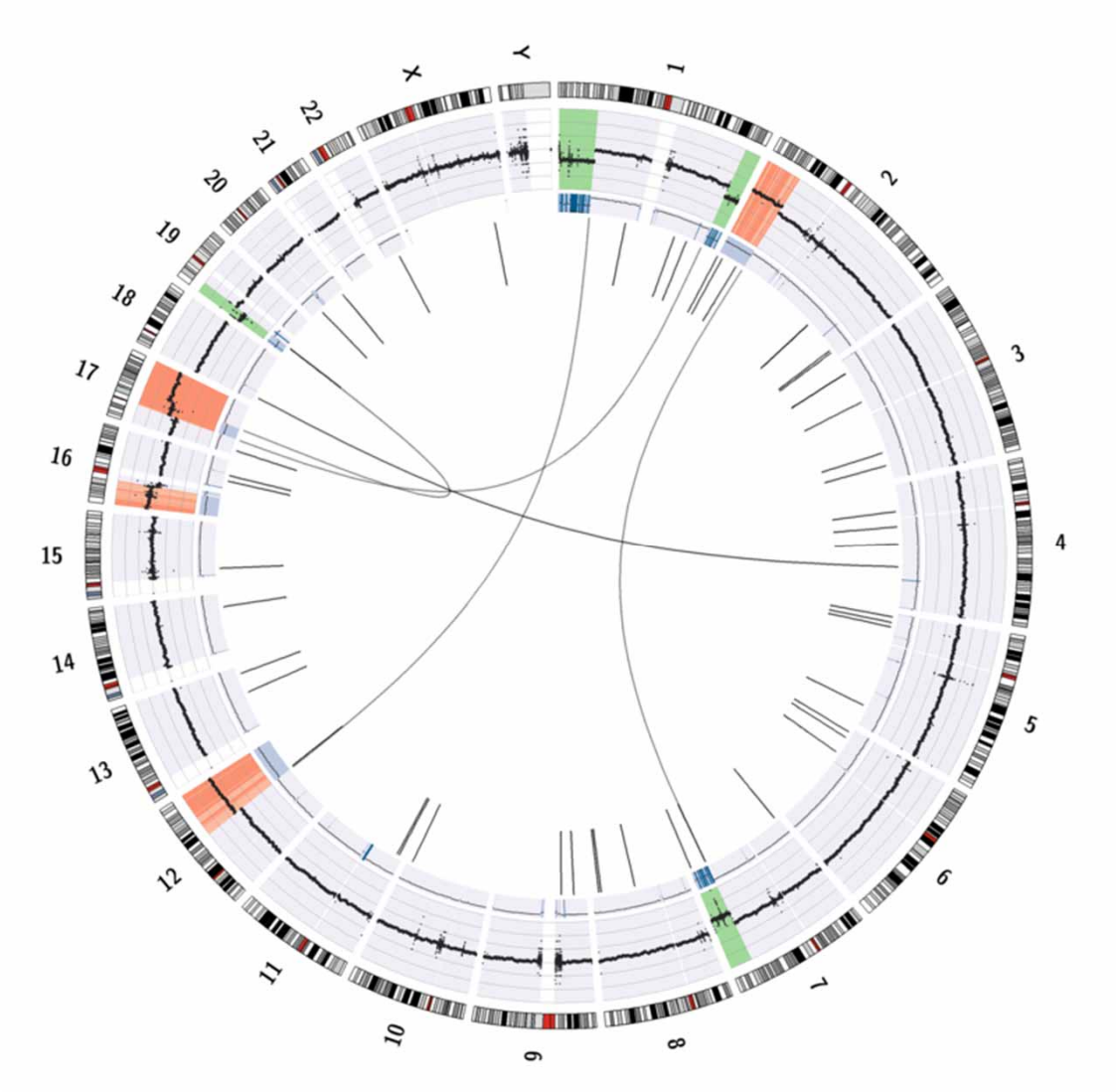


Paired-end sequencing to identify structural variants

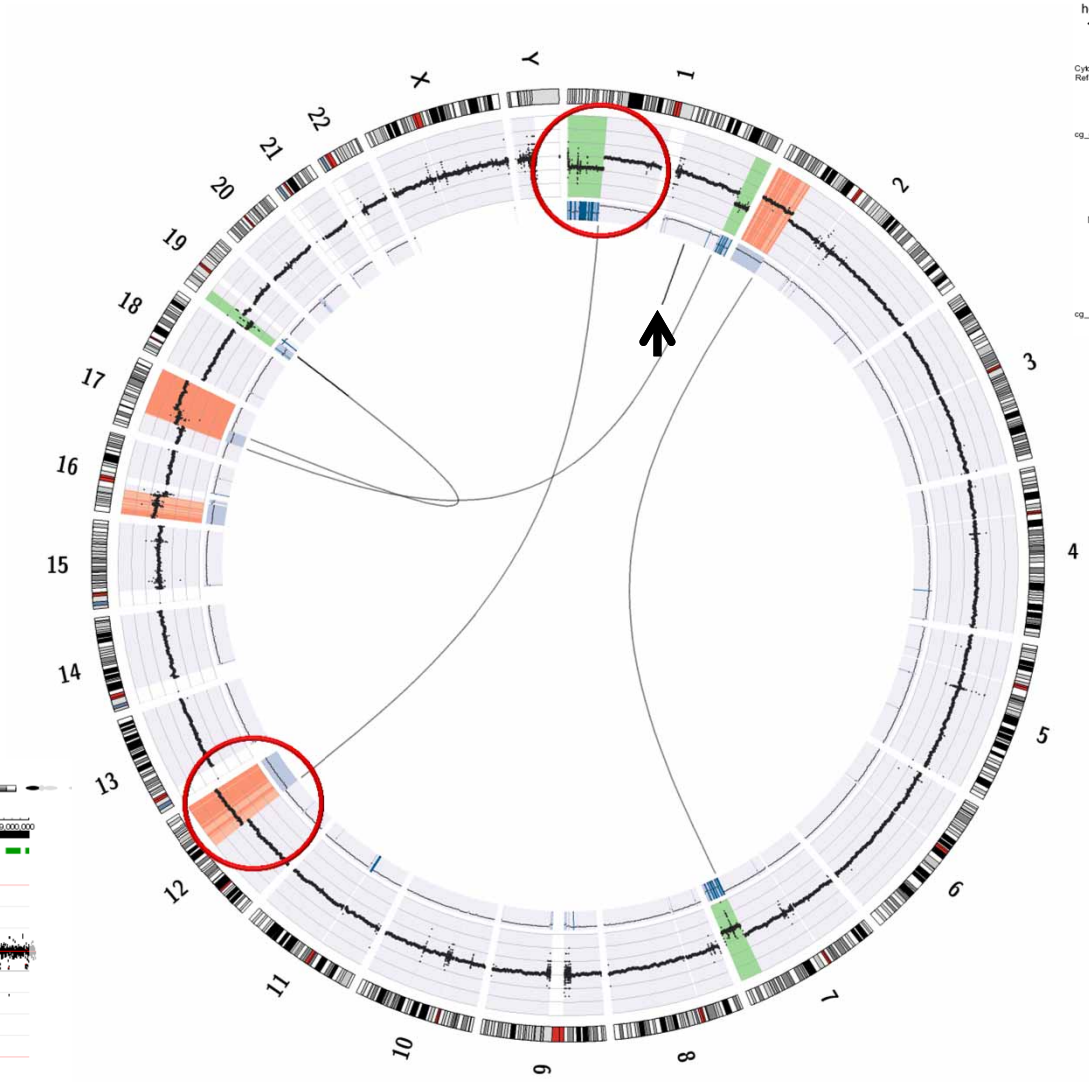


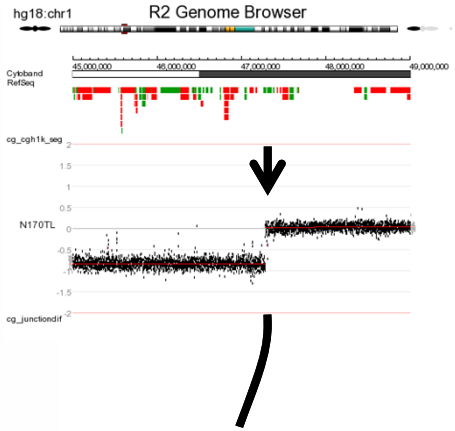
Event Type in sequenced genome	Manifestation when mapped to reference genome
Insertion	Mate distance << 500
Deletion	Mate distance >> 500
Inversion	Conflicting mate orientations
translocation	Conflicting mate chromosomes

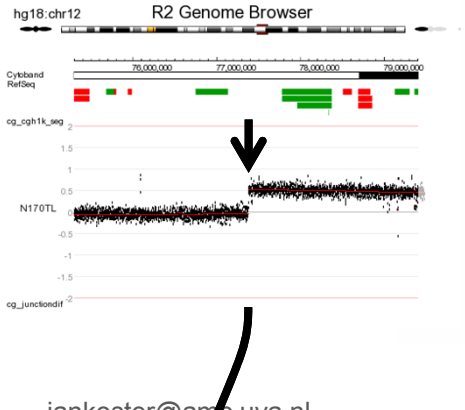
Structural variants



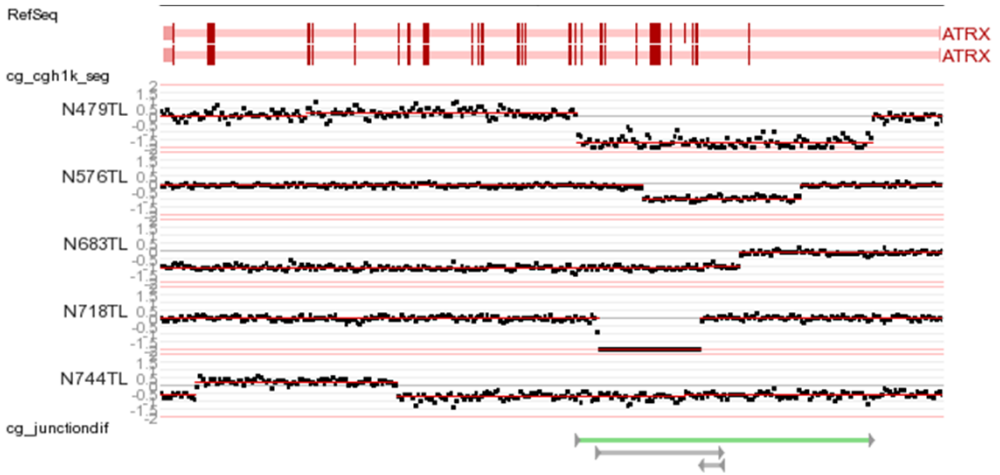
Somatic structural variants

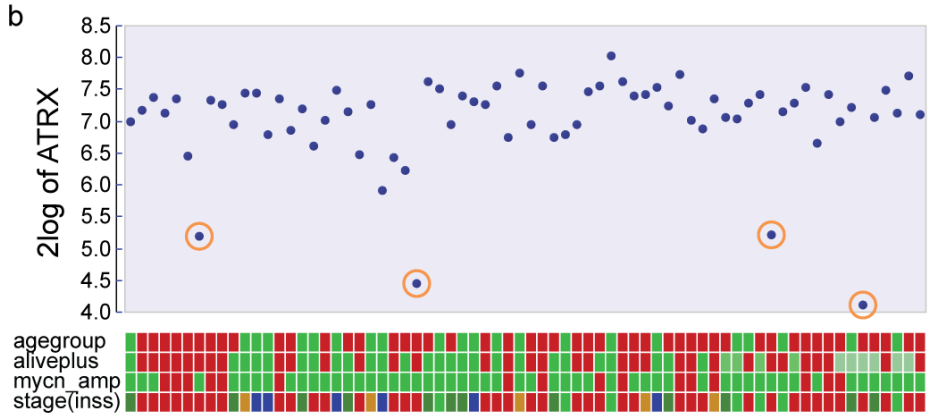






Structural variation affects single genes



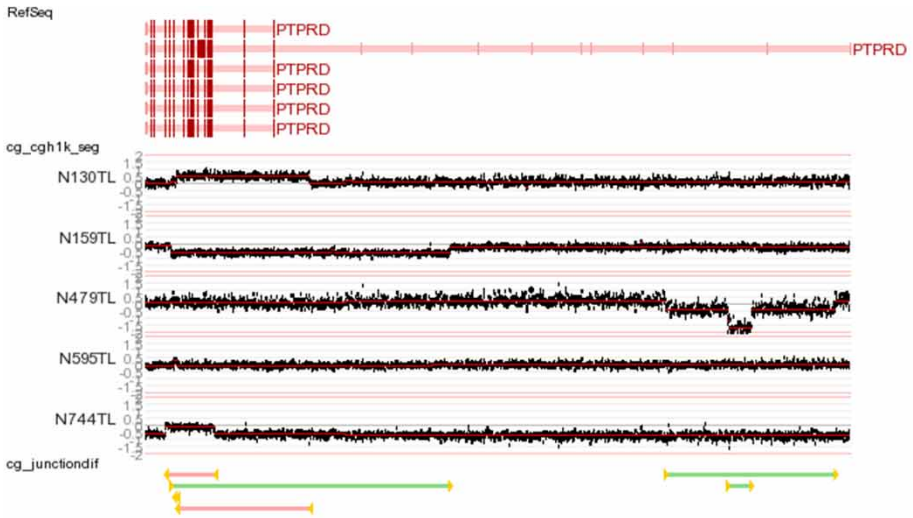


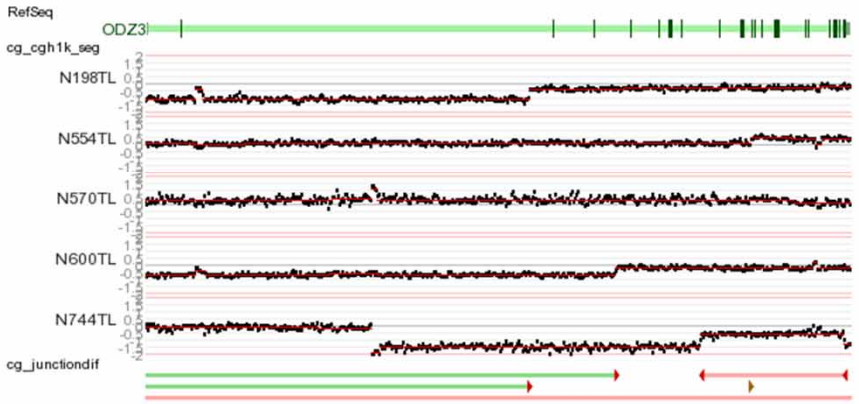
Molenaar & Koster et al, Nature, 2012

- ATRX gene
  - Broken -> Alternative lengthening of telomeres (ALT)
- Cheung et al, JAMA 2012
  - ATRX mutations in older neuroblastoma patients with dismal prognosis

	age at diagnosis (Y)
N479T	3.7
N576T	4.6
N683T	3.9
N718T	3.5
N744T	17.0

Structural variation affects single genes



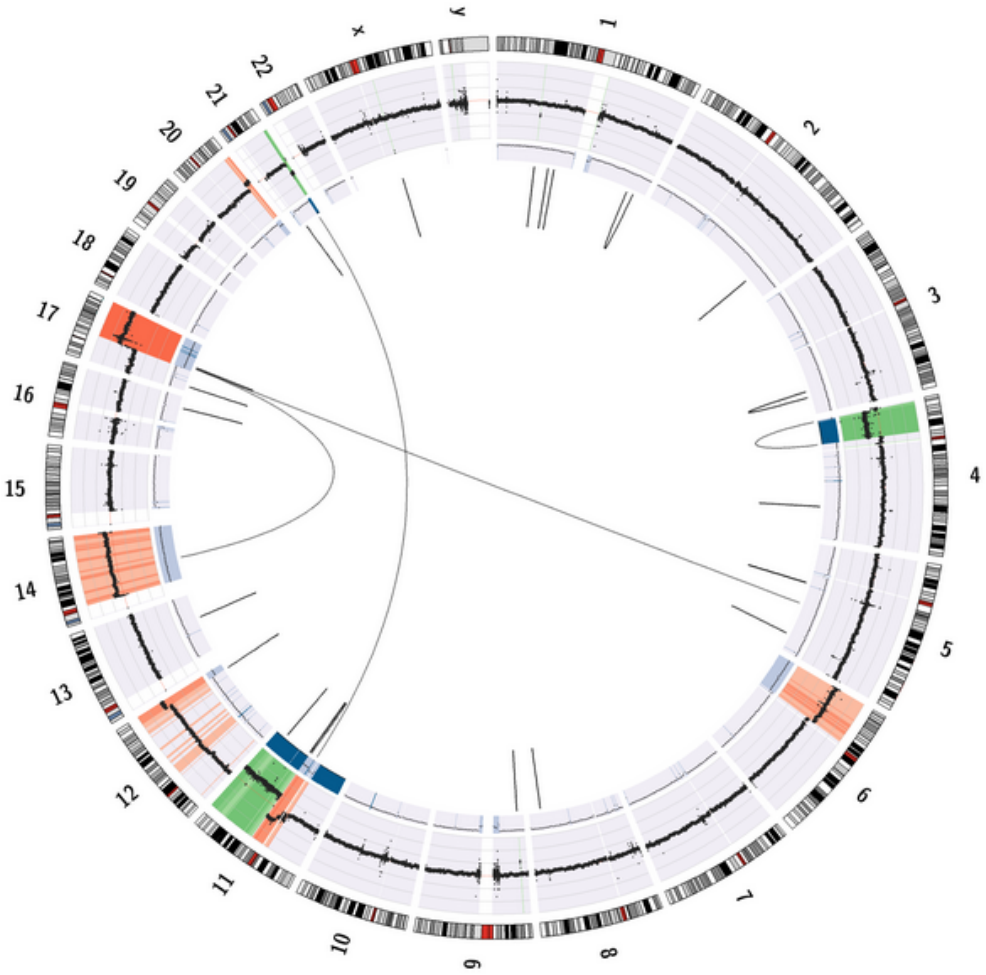


- PTPRD (5)
  - Involved in Growth Cones

- ODZ family (10)
  - Axon Guidance / Neuritogenesis
  - ODZ3: 5 structural defects
  - ODZ2: 2 missense defects
  - ODZ4: 1 structural + 2 missense

Molenaar & Koster et al, Nature, 2012

Structural variation affects single genes



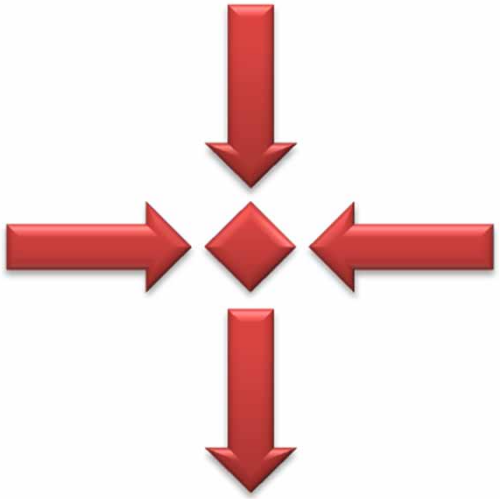
- **Select events that affect limited number of genes**
  - **Deletion/duplication within 1 gene**
  - **Inversions**
  - **Potential fusion genes**

451 events

Integration of mutation and SV data

Splice junctions (n=37)
Variants within 2 bases around the exon-bounderies

Somatic mutation (n=586 genes)
Amino change or worse
Keep all validated and true
Remove validated and NOT true
Somatic>0.1
Not in CG reference samples
Not in lymphocytes
No insertions or substitutions
If a gene has 0% present calls in nb119 but >0% in normal504, then skip



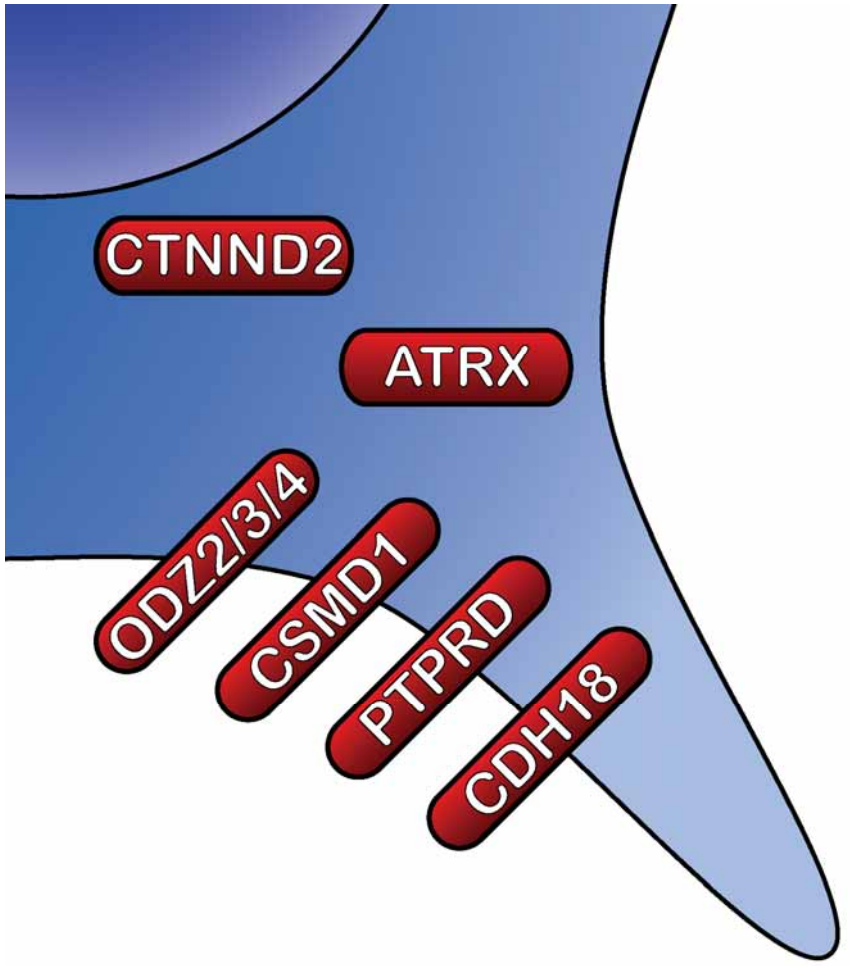
Somatic Structural Variants (n=451)
(no baseline genome_v2; no under represented Repeats; length>=70; mates>=10; no artifact)
Break by inversion
Exon bites
<=5 expressed genes in region <=1,000,000
Potential fusion strand match

Combined table (n=1,041)

Integration of mutation and SV data

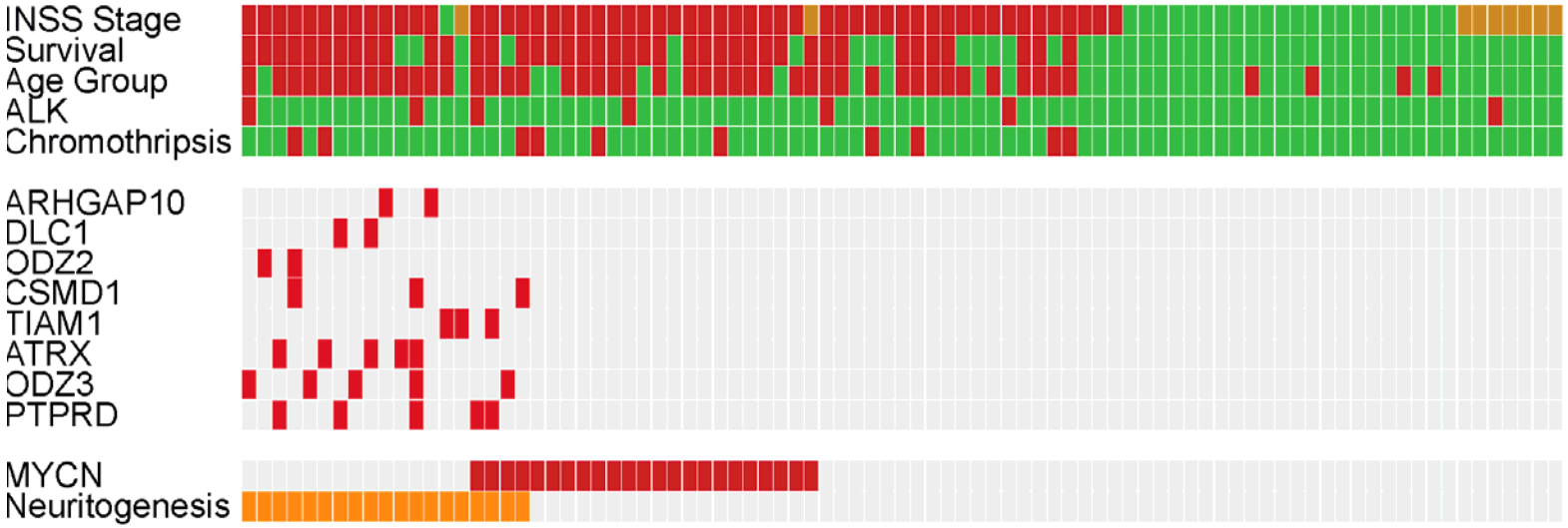
Recurrent gene defects:

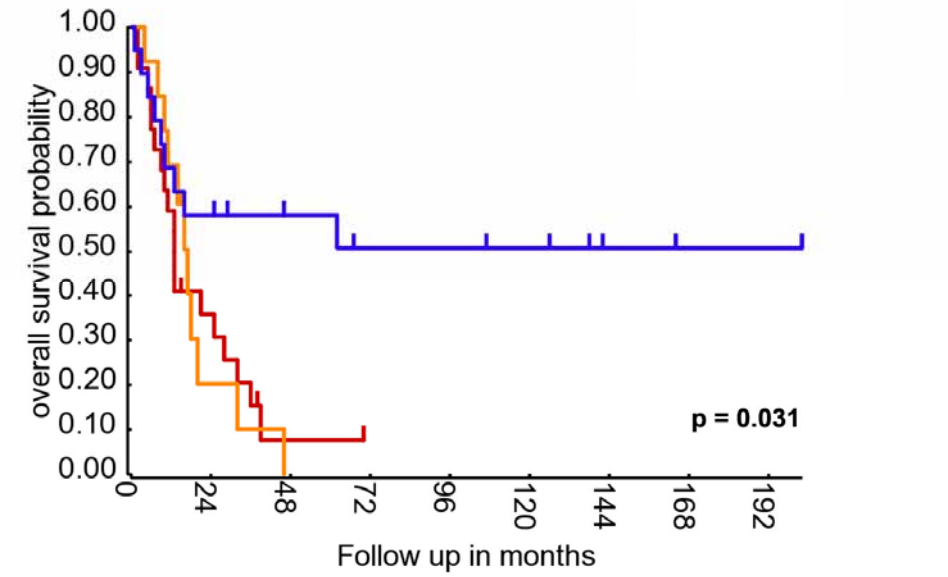
- 11 genes >=3 patients
- Most recurrent genes involved in neuritogenesis / growth cones
  - PTPRD / ODZ3



Many of the recurrent genes have also been identified in other sequencing efforts

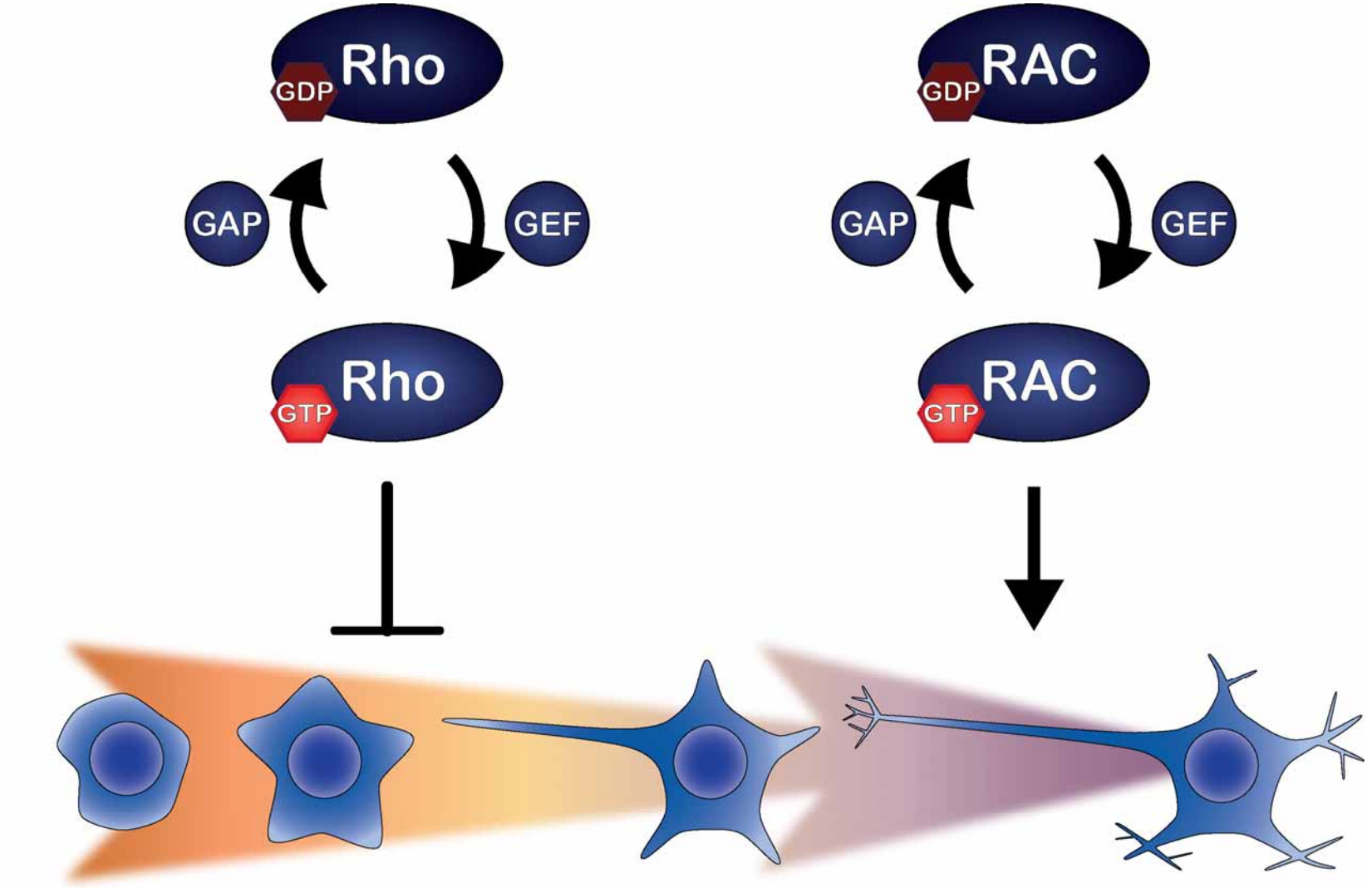
Neuritogenesis defects cluster in high stage Neuroblastoma





Molenaar & Koster et al, Nature, 2012

RAC/Rho signaling in neuritogenesis



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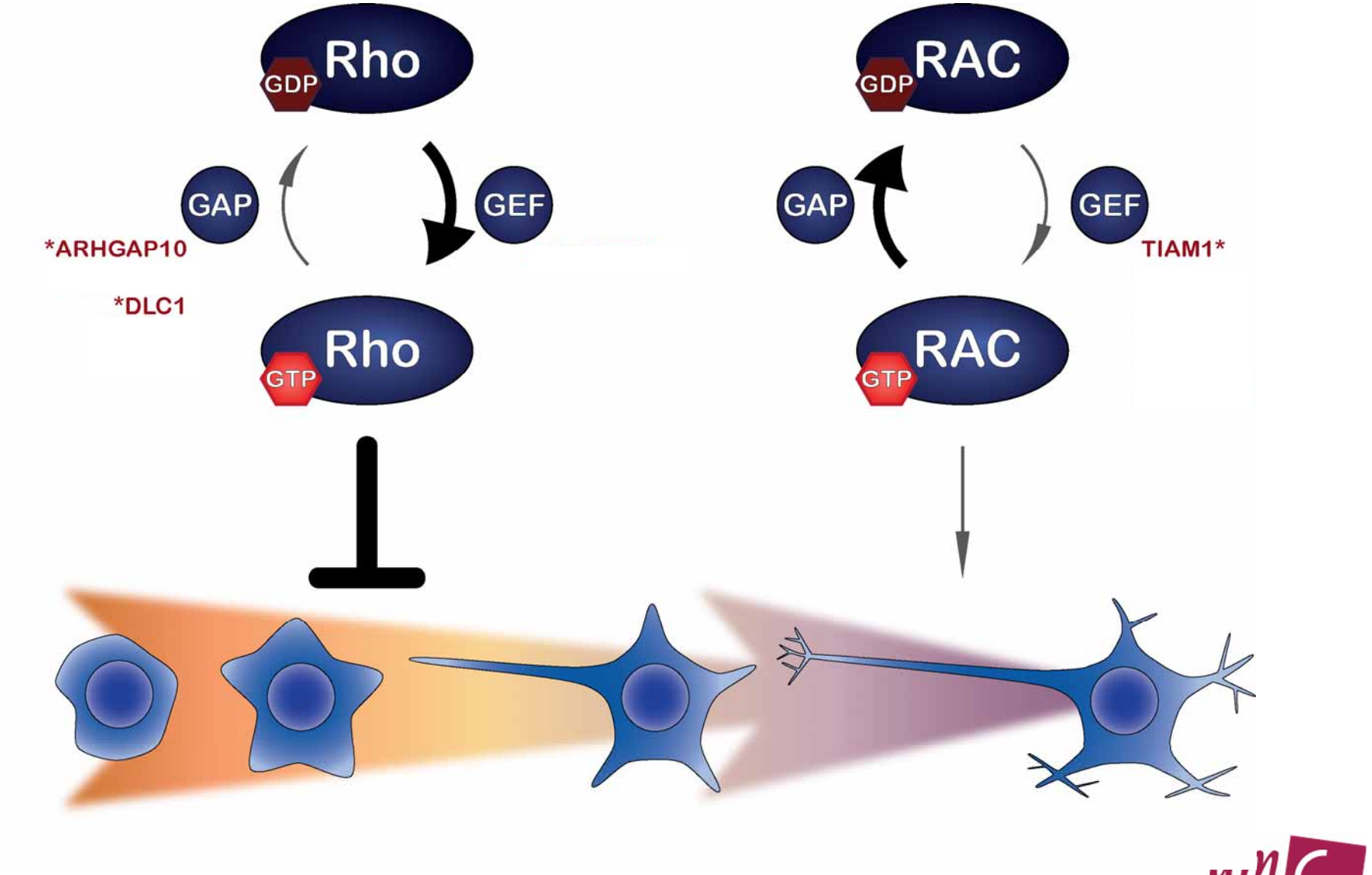

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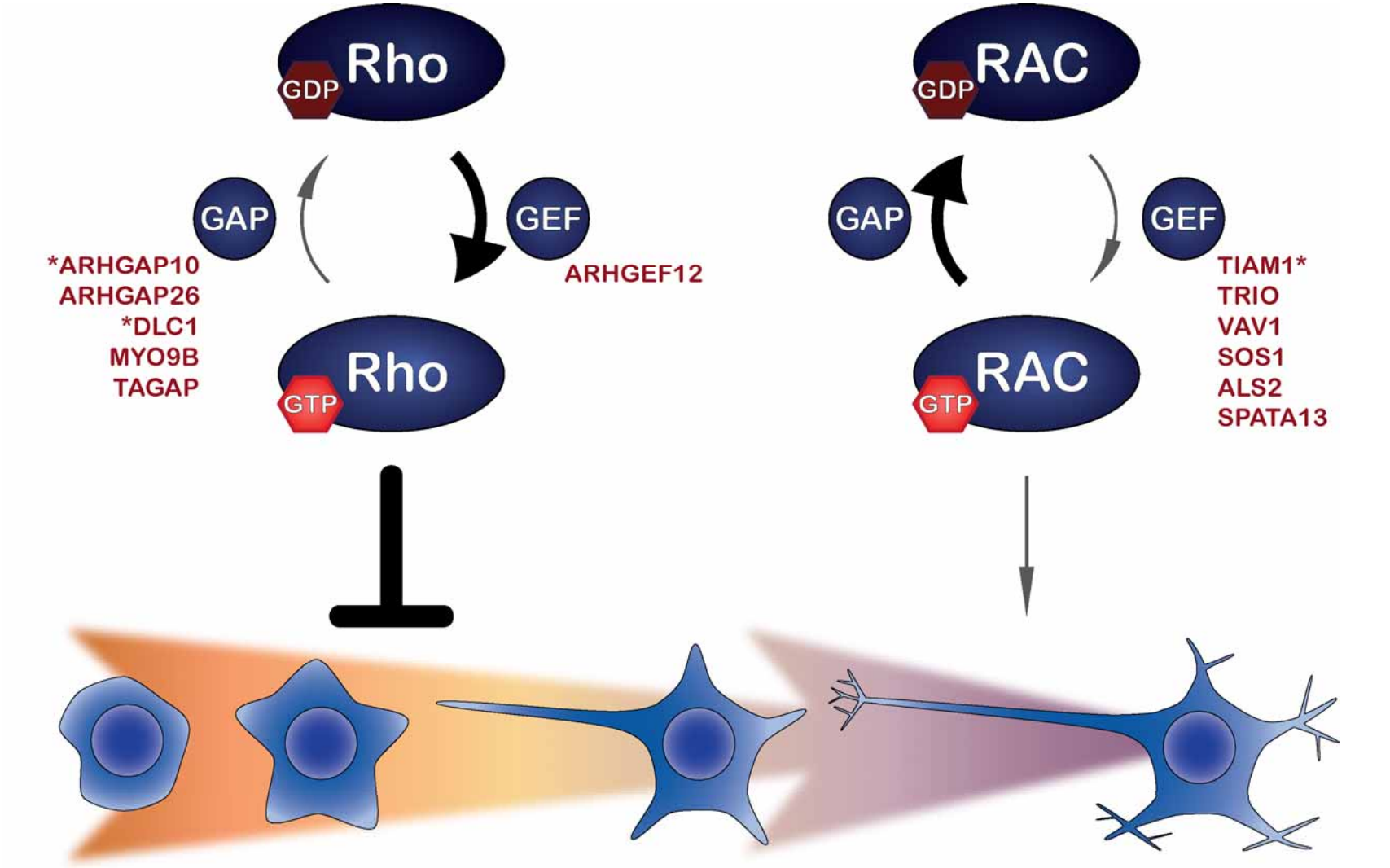
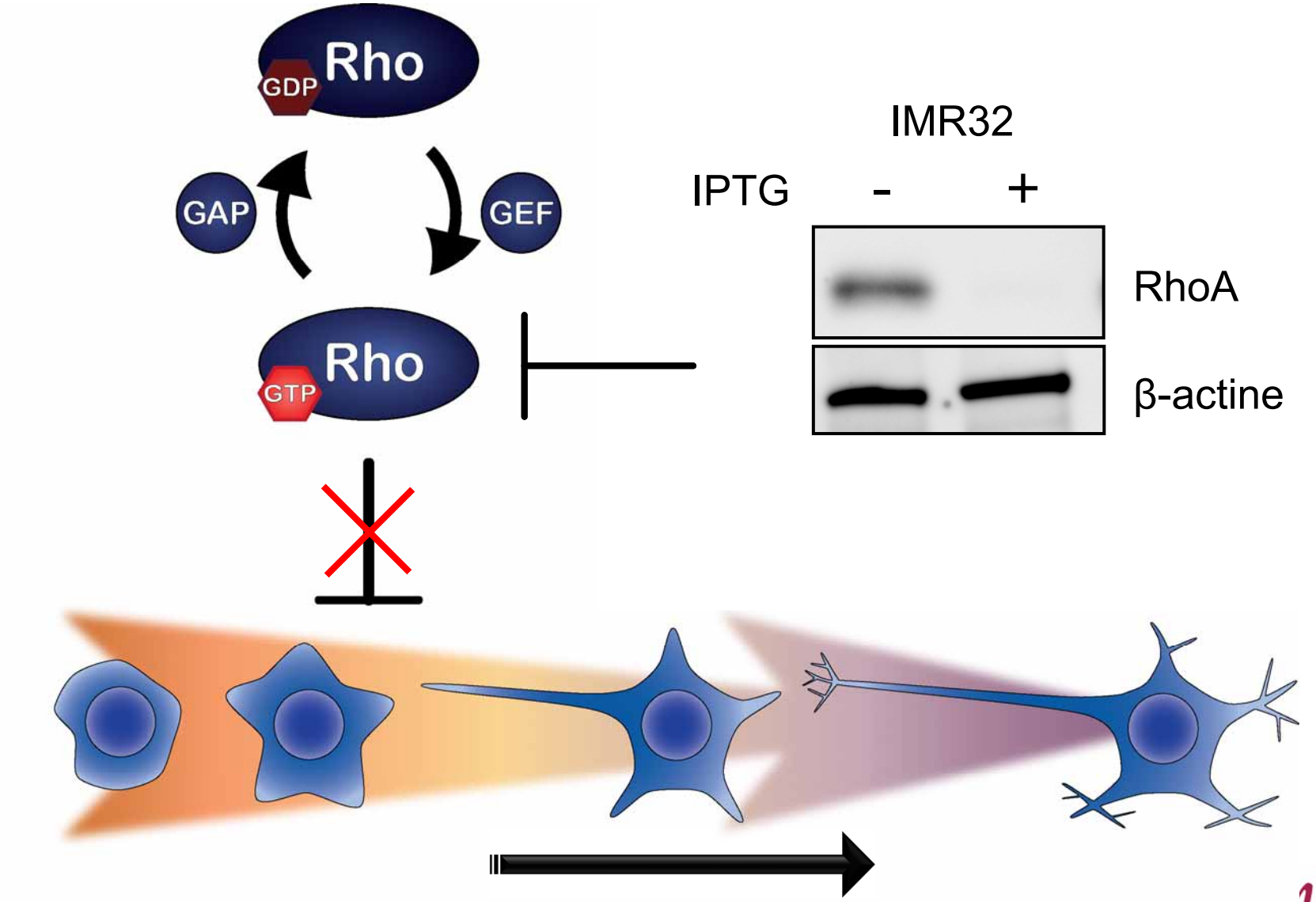


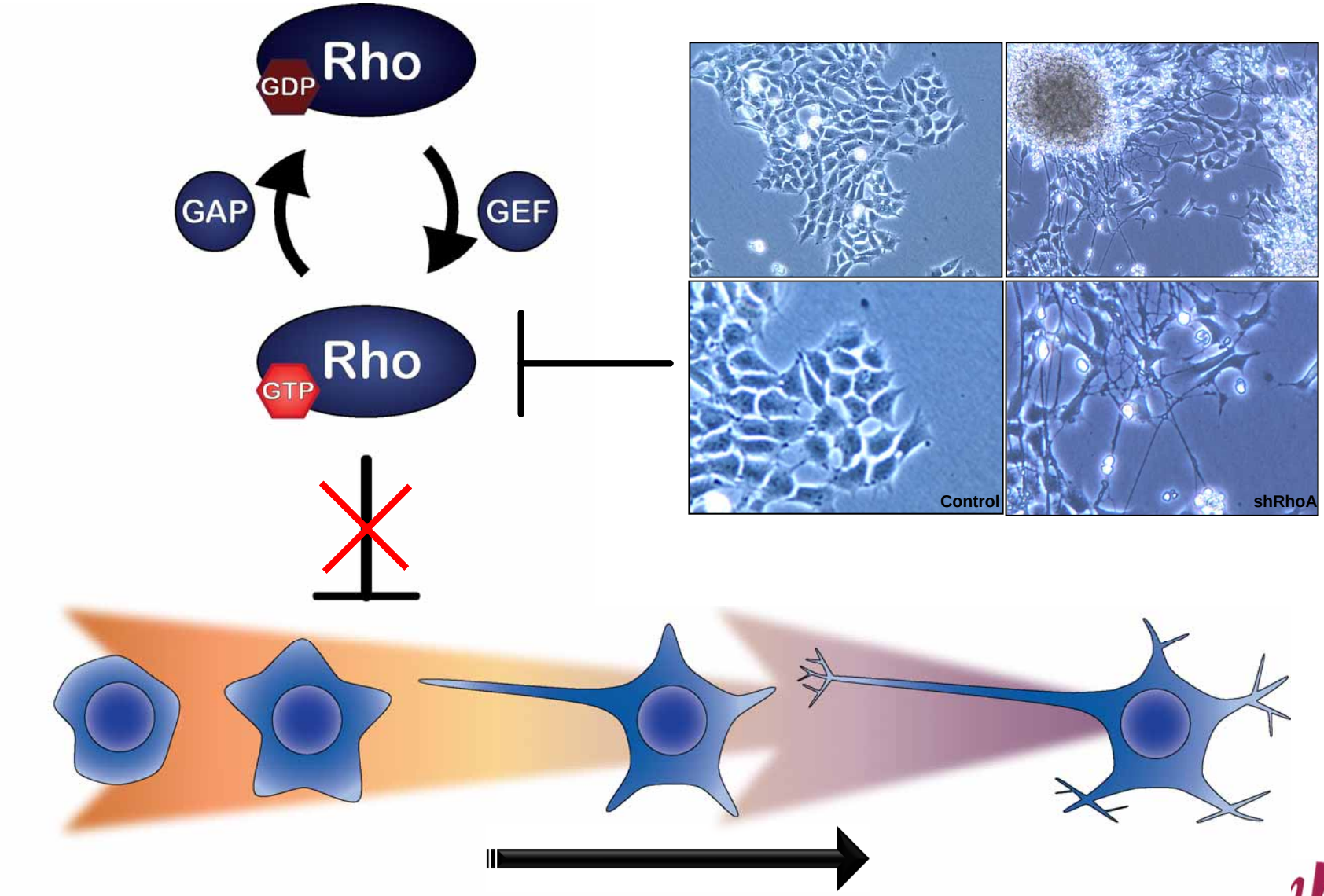
p=0.025 (Fisher's Exact)

Inducible RhoA knockdown



Ellen Westerhout

Inducible RhoA knockdown



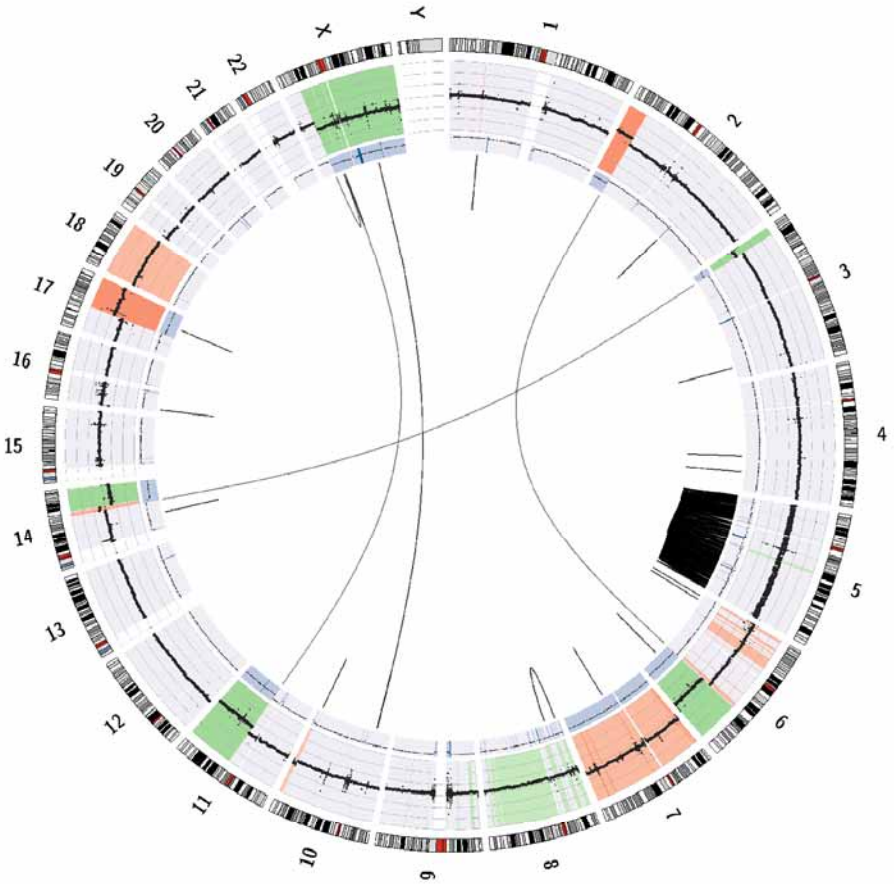
Ellen Westerhout

Conclusions from WGS of neuroblastoma

1 Low frequency of somatic mutations

2 Neuritogenesis signaling frequently affected in high stage neuroblastoma

Chromosome shattering in neuroblastoma



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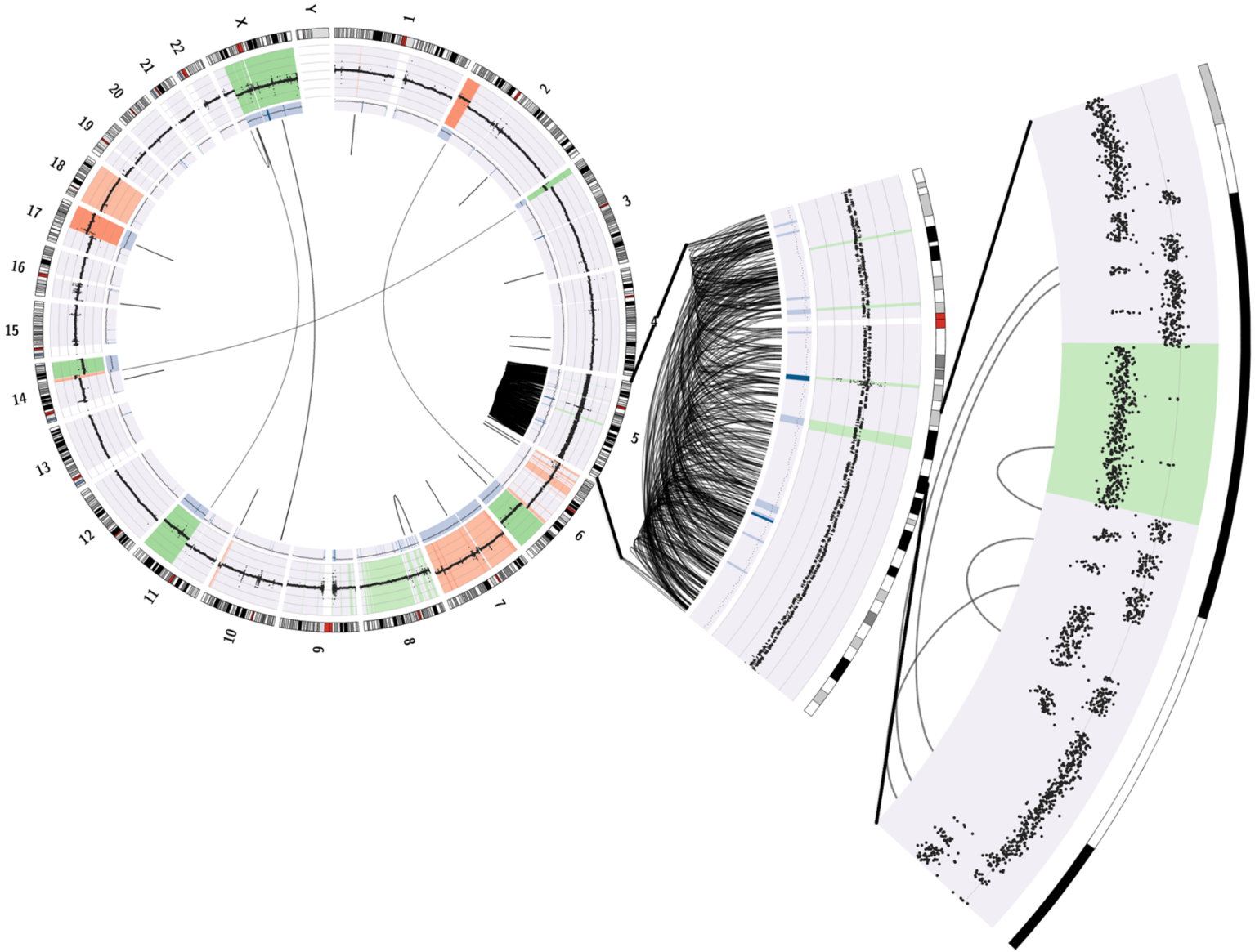

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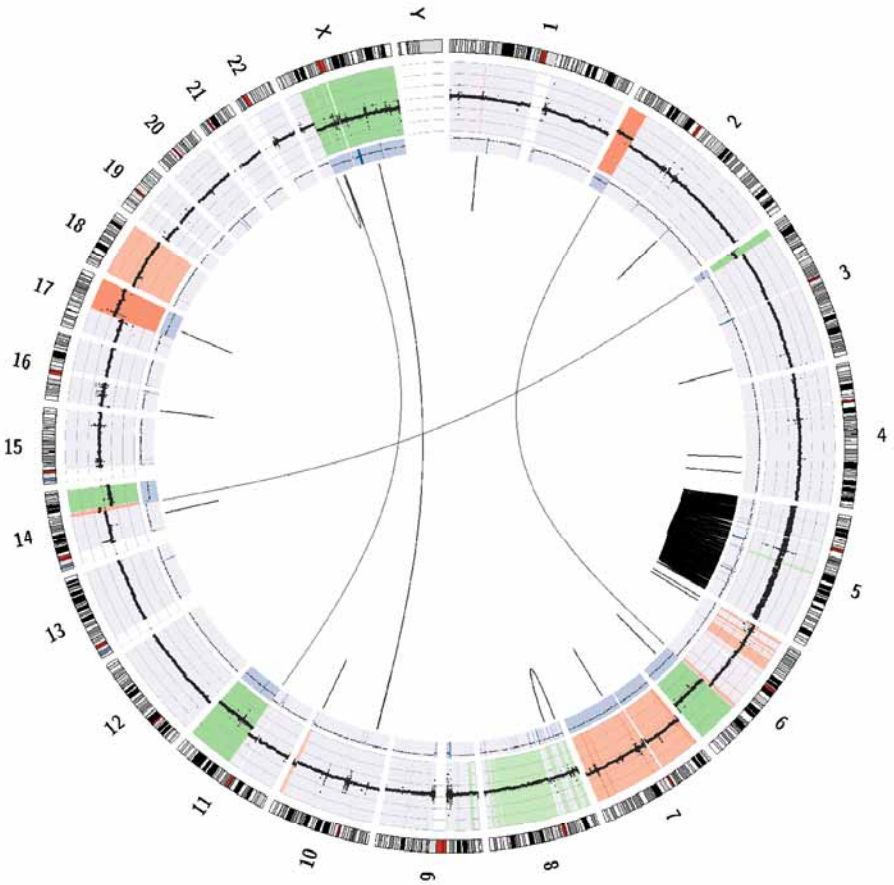
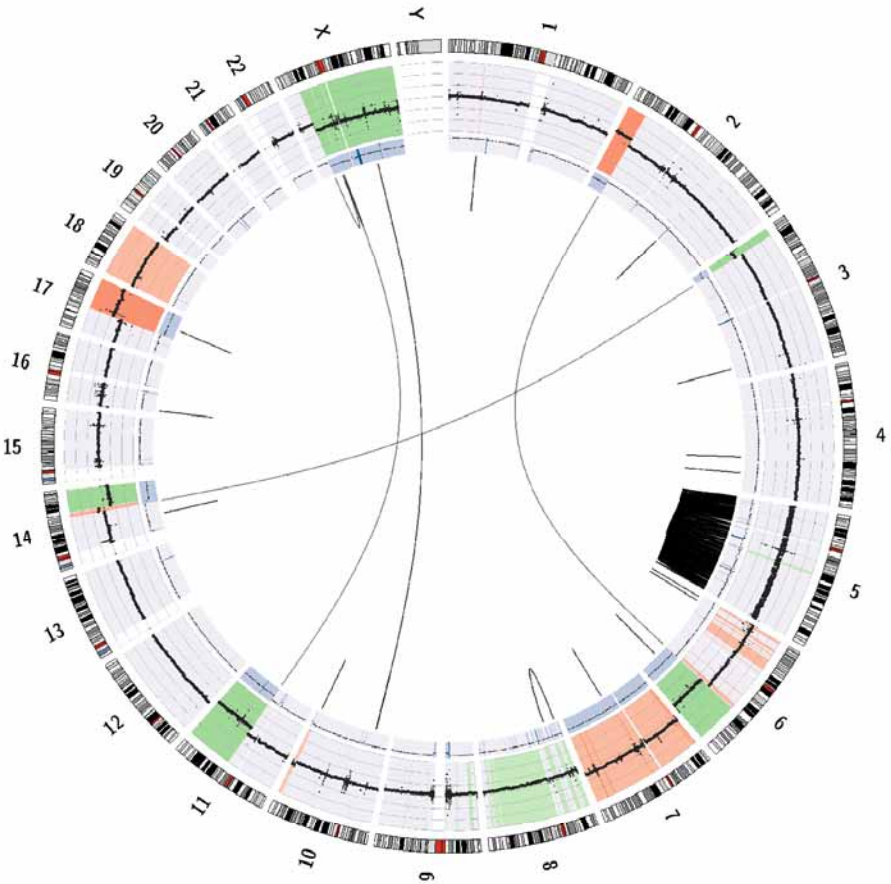
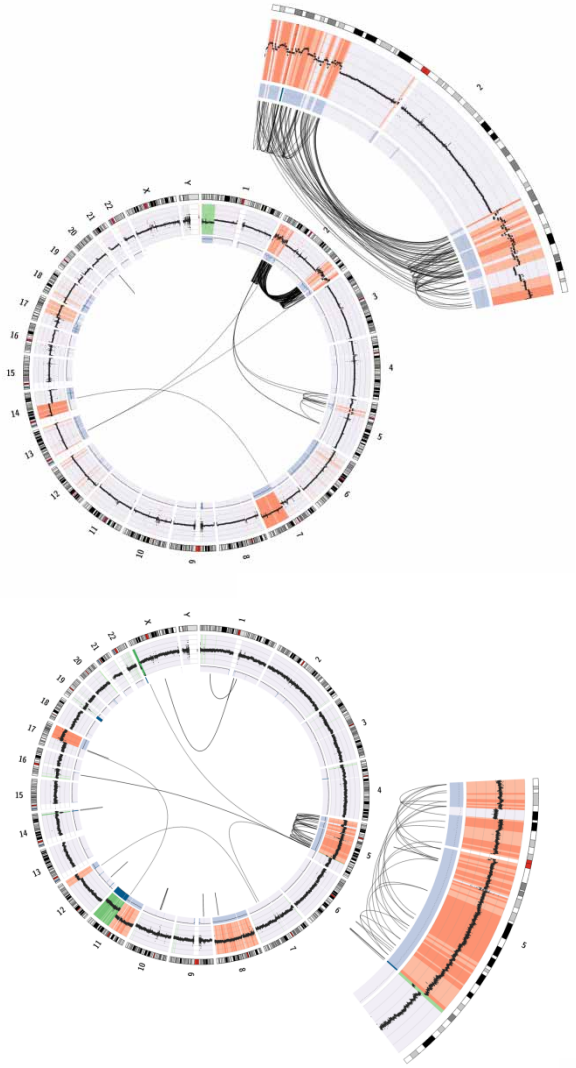


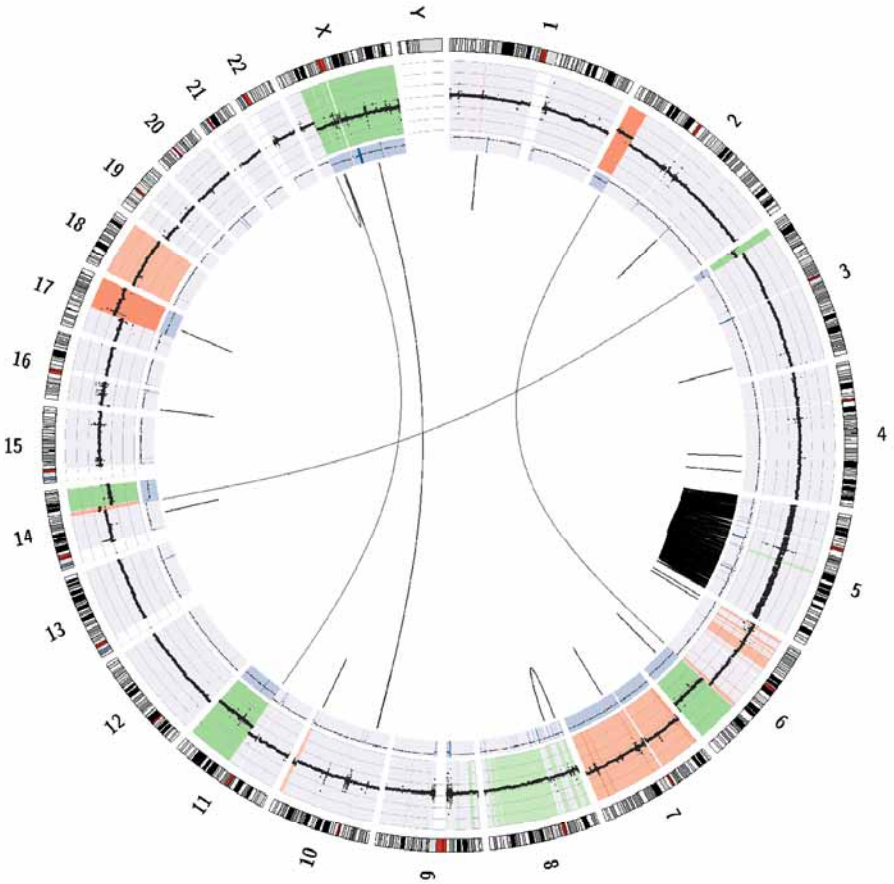
- Chromothripsis (2011)
  - Greek for 'chromosome' (*chromo*) and 'shattering into pieces' (*thripsis*)
  - Single catastrophic event (limited state changes)

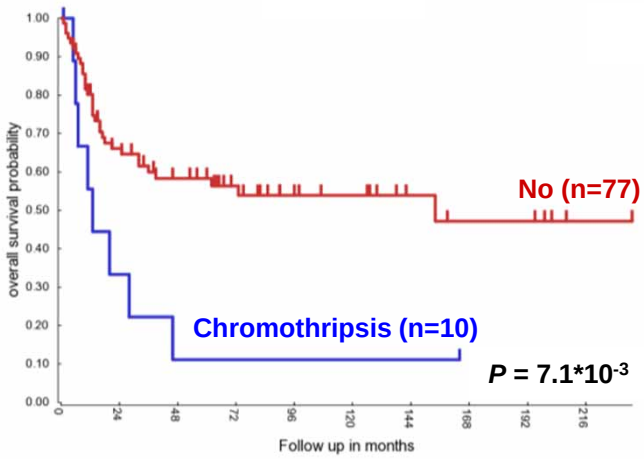
Chromothripsis in neuroblastoma



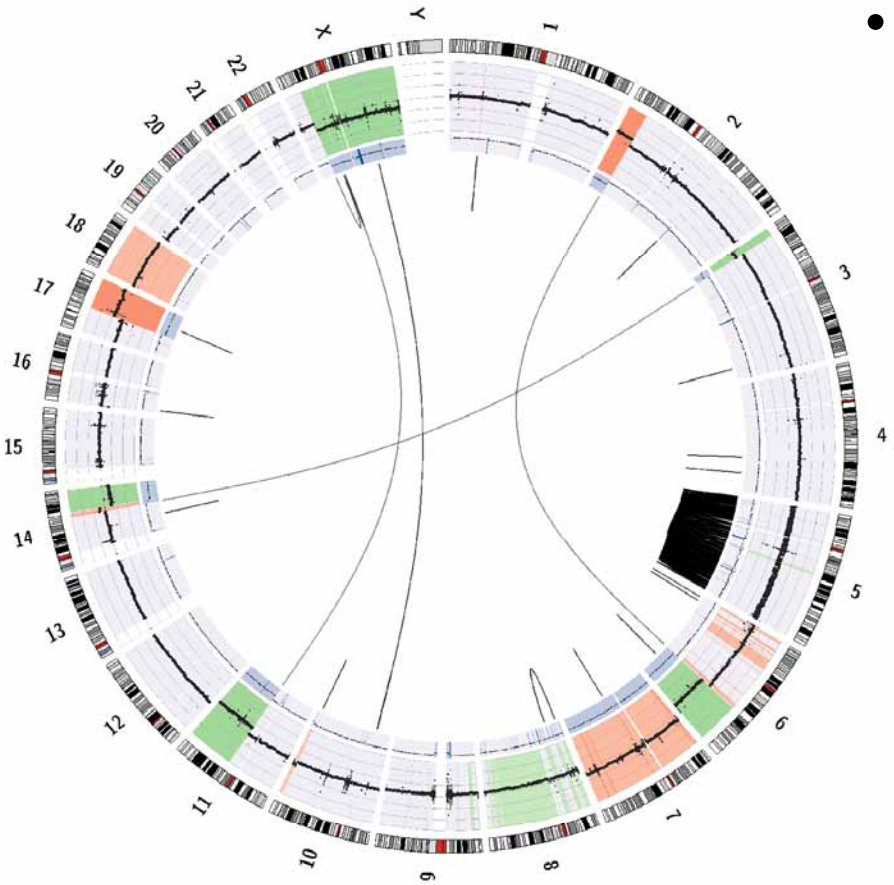


Chromothripsis in neuroblastoma





Chromothripsis in neuroblastoma



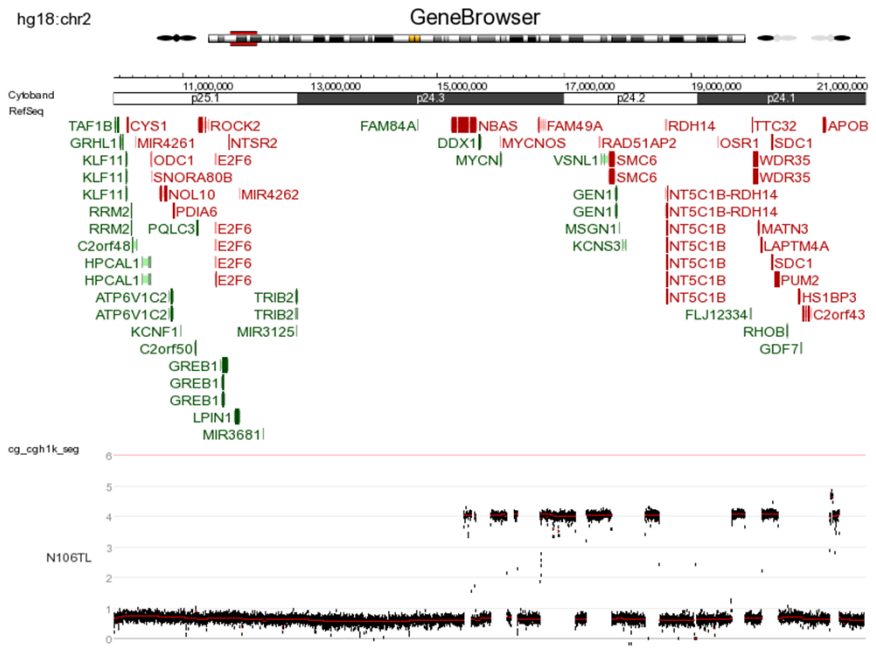
- 10 cases
  - All high stage (18% of high stage)
    - 9 times inss 4
    - 1 times inss 3
  - 4 patients MYCN amplification
  - 8 patients diseased
  - 2 cases patient derived cell lines are being maintained

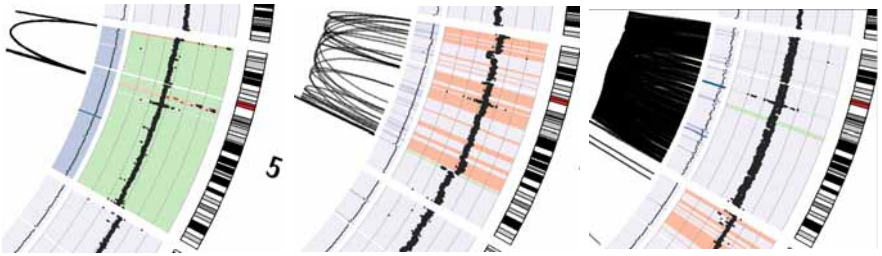
cause/mechanism chromothripsis elusive

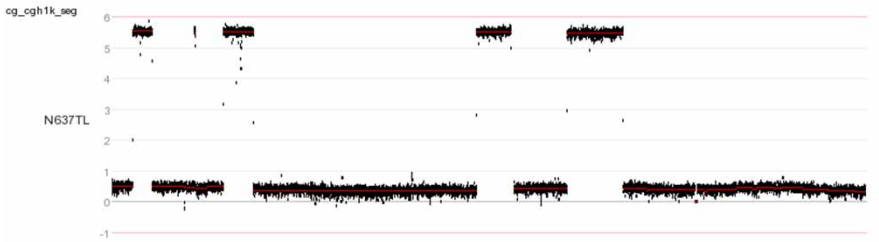
- Limited state changes
  - Single event
- Usually localized to chromosome or region
  - Most likely when chromosomes are condensed (mitosis)
- Micronuclei
  - Occur upon segregation errors
  - Shown to generate structural chromosome alterations

Potential targets of chromothripsis

- Genes known to be of importance to NB
  - MYCN
  - MYC
  - CDK4
- Also number of unknowns
  - Chromosome 5

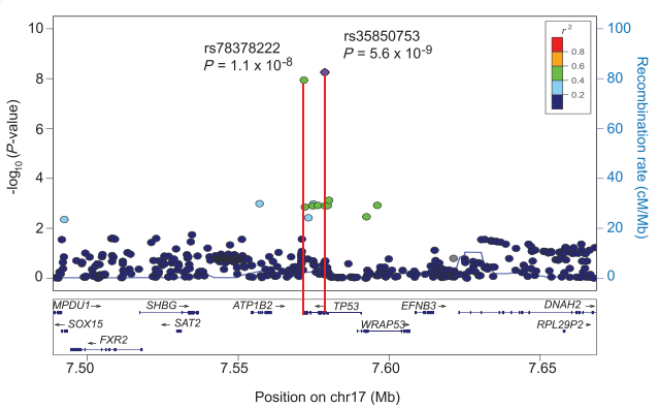




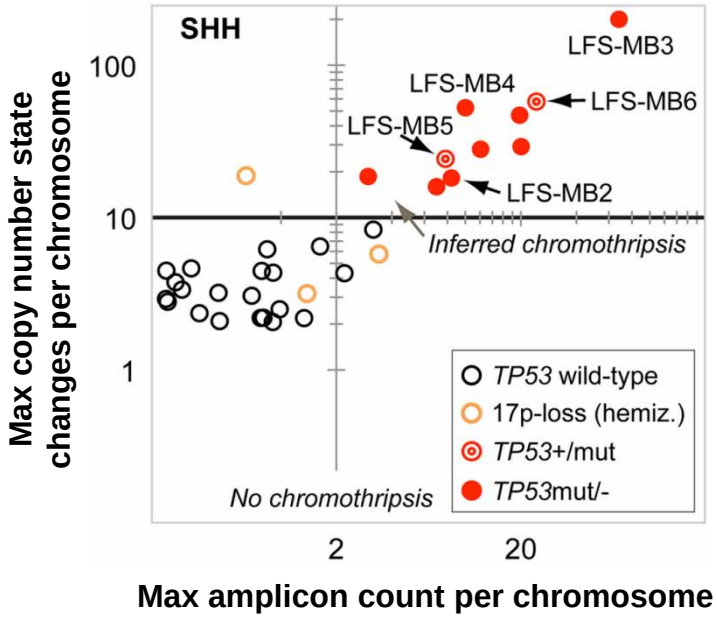


Associations with chromothripsis

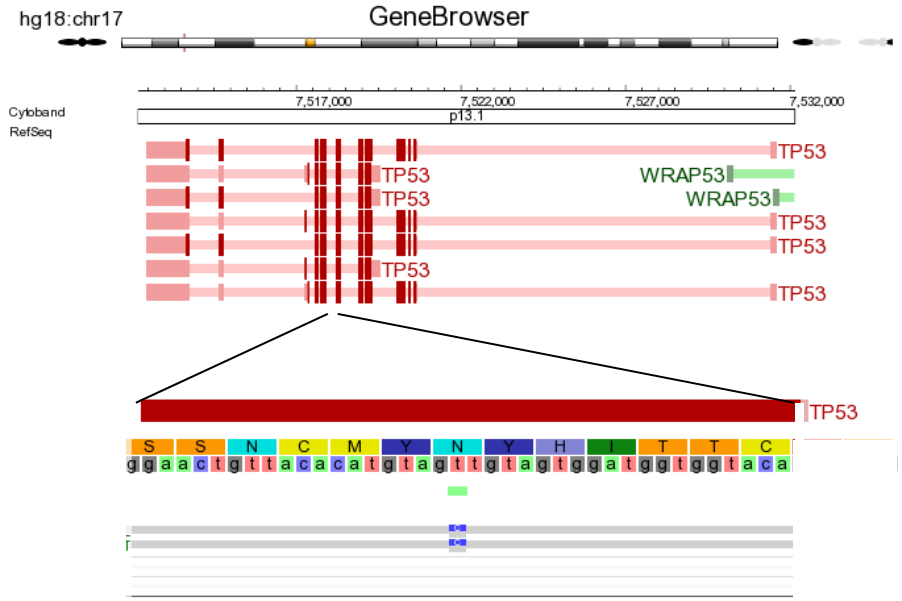
- Rausch et al, Cell, 2012
  - SHH subtype of medulloblastoma
  - Frequent chromothripsis
  - Ass. with germline p53 mutations



Diskin et al, JNCI, 2014

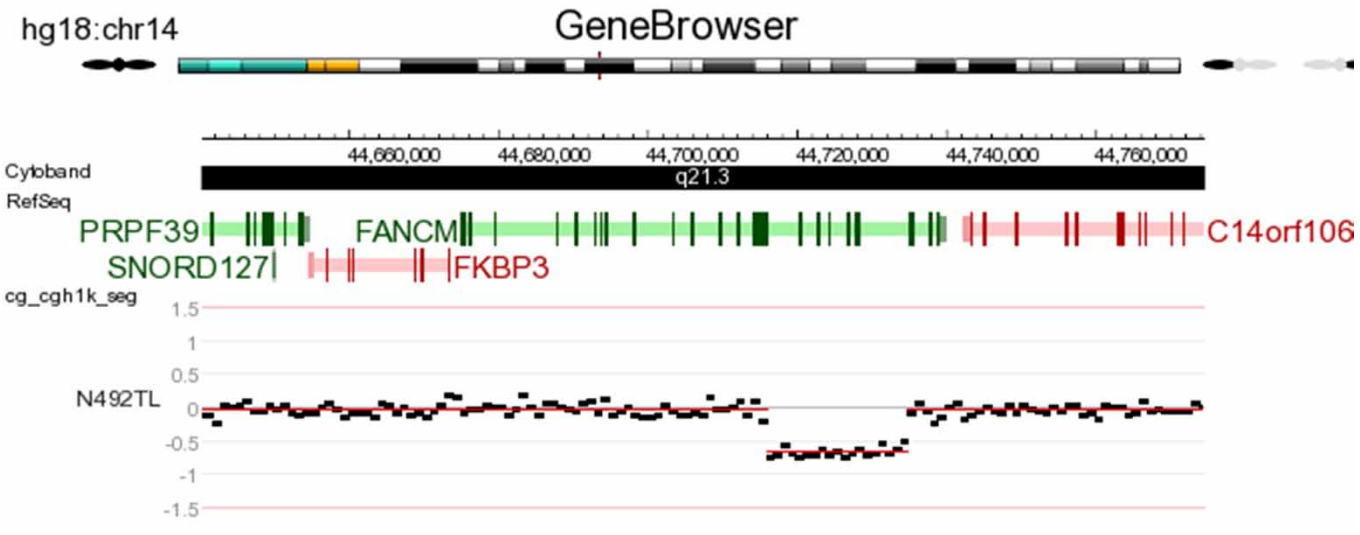


Rausch et al, Cell, 2012



Associations with chromothripsis

- FANCM microdeletion
  - DNA damage response
    - Stalled replication forks



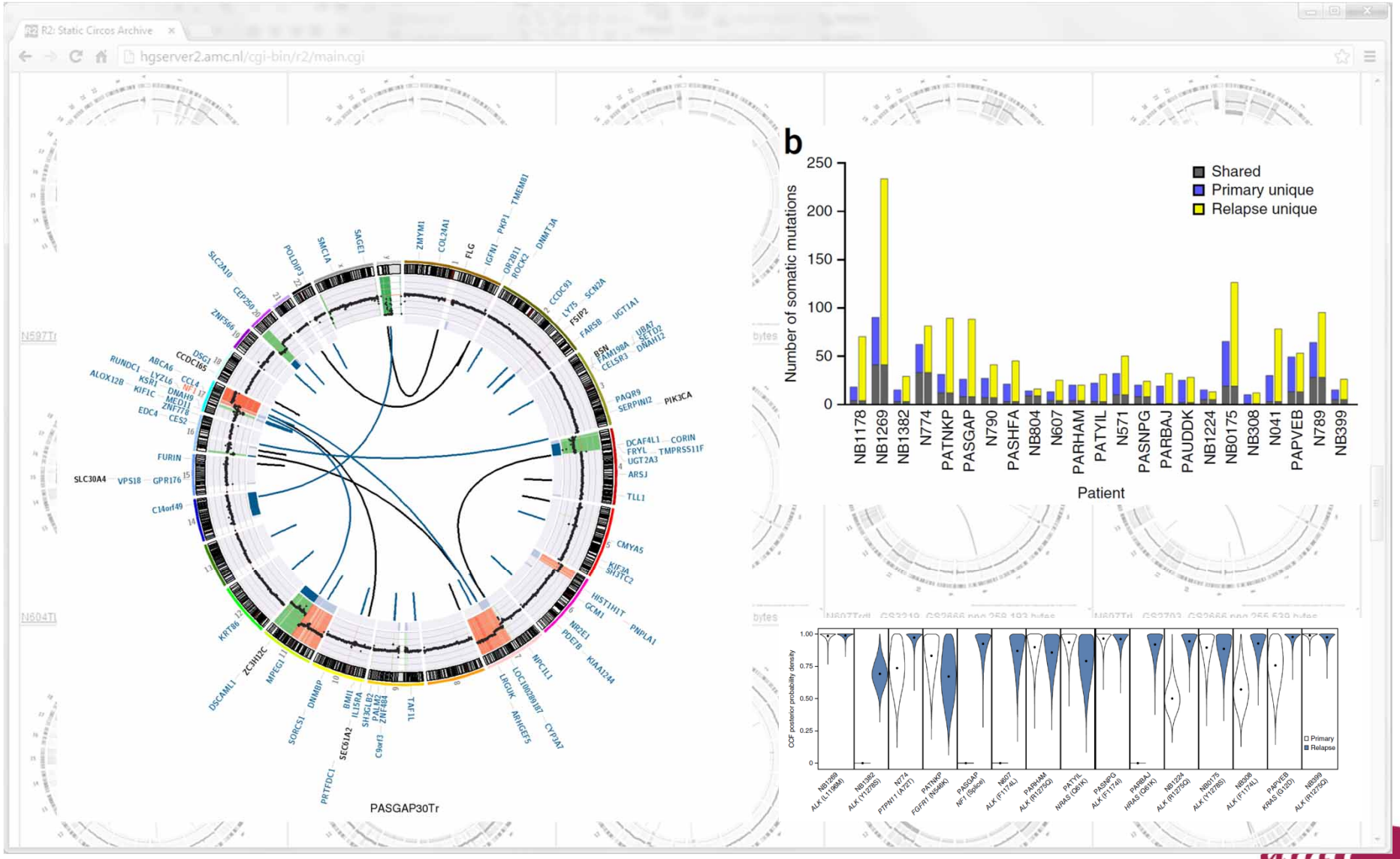
Conclusions from WGS of neuroblastoma

1 Low frequency of early tumor driving mutations

2 Neuritogenesis signaling frequently affected in high stage neuroblastoma

3 Chromotripsis associated with high stage neuroblastoma, poor prognosis and aberrations in DNA damage signaling

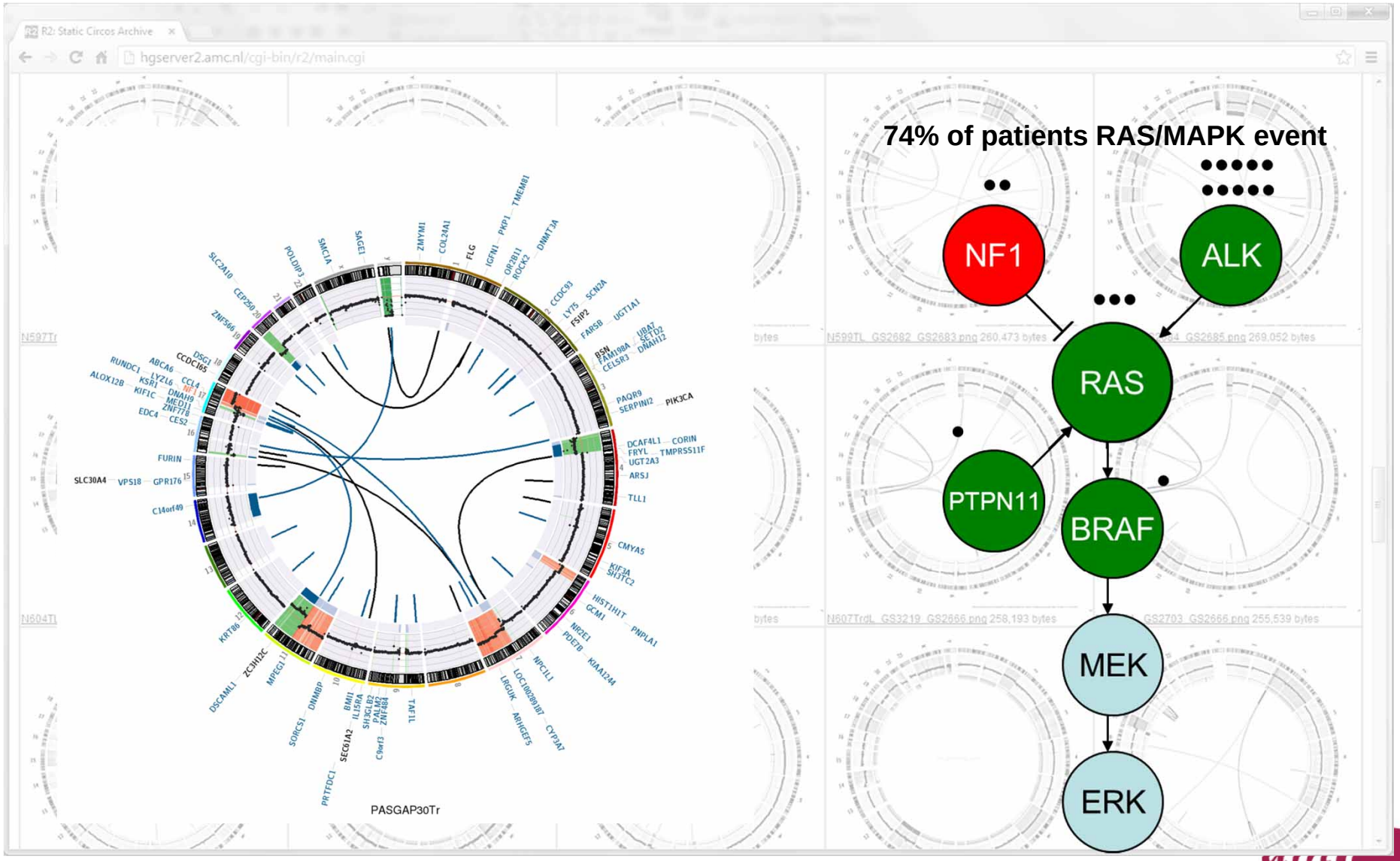
Neuroblastoma relapses (n=23 trio's)



Eleveld et al, Nature Genetics, 2015

Onco Genomics

Neuroblastoma relapses (n=23 trio's)



Eleveld et al, Nature Genetics, 2015

Onco Genomics

Conclusions from WGS of neuroblastoma

1 Low frequency of early tumor driving mutations

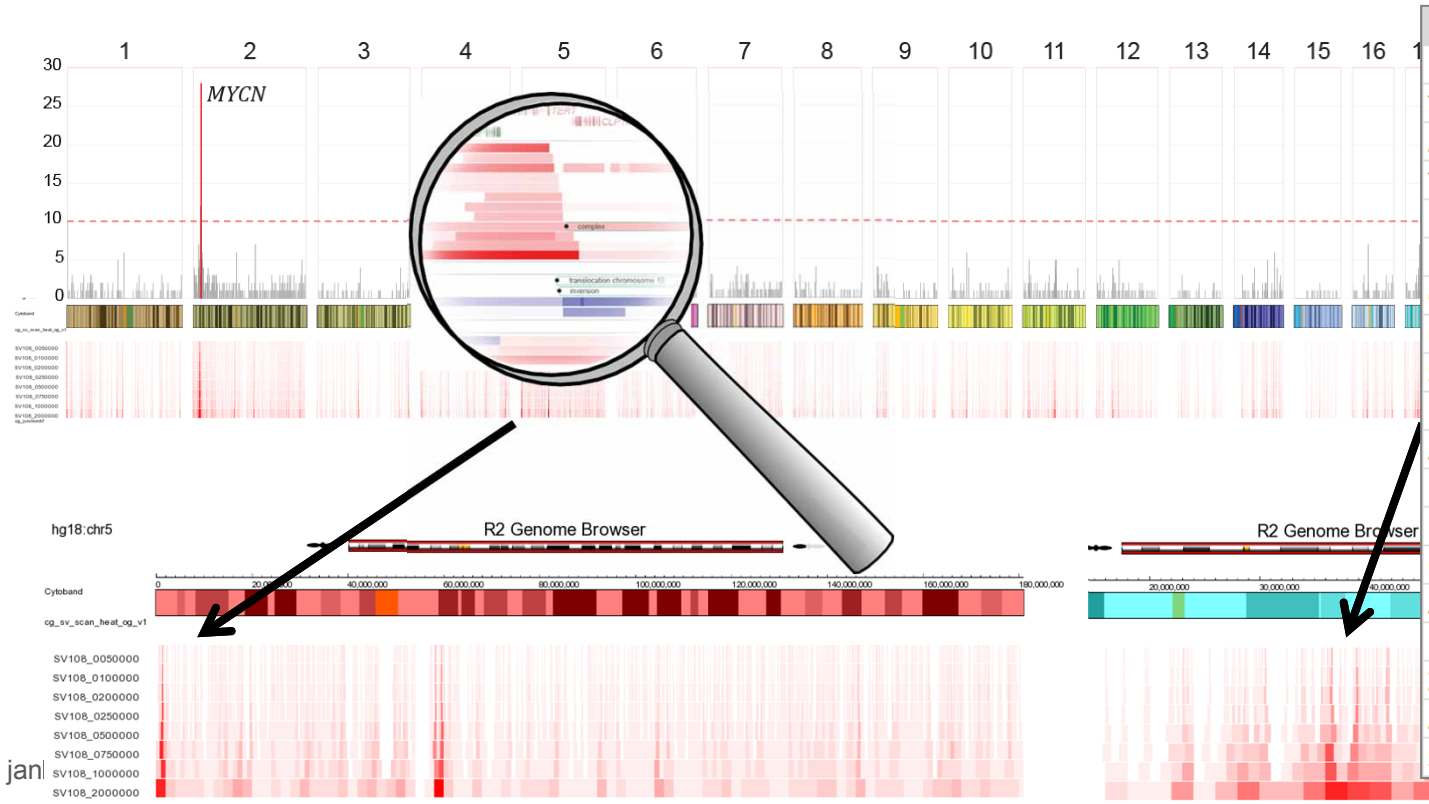
2 Neuritogenesis signaling frequently affected in high stage neuroblastoma

3 Chromotripsis associated with high stage neuroblastoma, poor prognosis and aberrations in DNA damage signaling

4 Relapsed neuroblastoma landscape different from primary tumors and reveals new targets for therapy

Survey Structural Variation

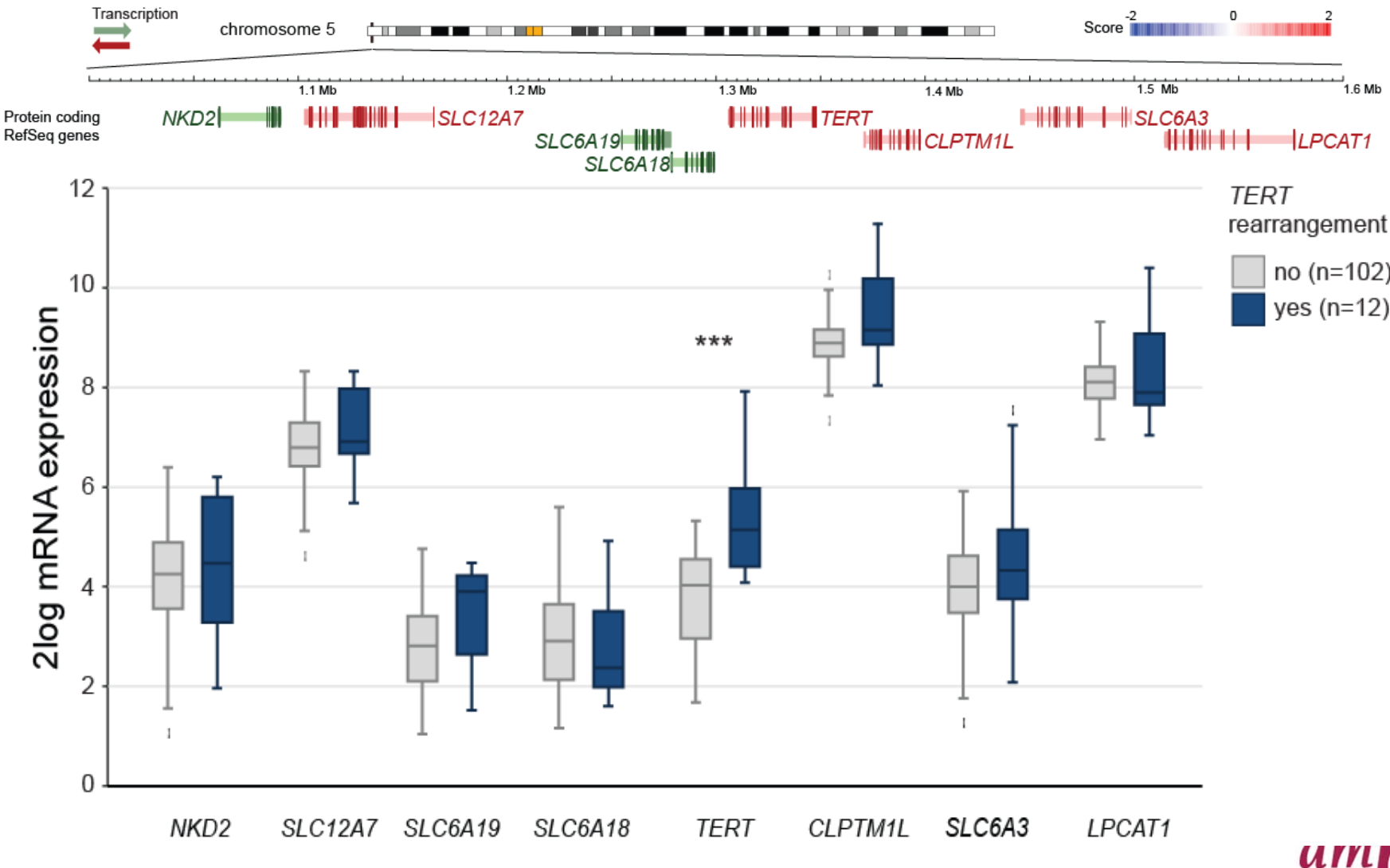
- Are there hotspots of SV in the neuroblastoma genome?



Gene	#_samples	Where
MYCN	28	SURR
TERT	13	US
ATRX	8	INTRA
TENM3	6	INTRA
PDE4D	6	INTRA
PTPRD	6	INTRA
GATA3	5	DS
CCND1	5	US
SHANK2	5	INTRA
PSEN1	5	US
ASCC2	5	INTRA
EIF4E	5	DS
MAML3	4	INTRA
CSMD2	3	INTRA
ADAM12	3	DS
UNC79	3	INTRA
ZFHX3	3	INTRA
ASIC2	3	INTRA
STAG2	3	INTRA

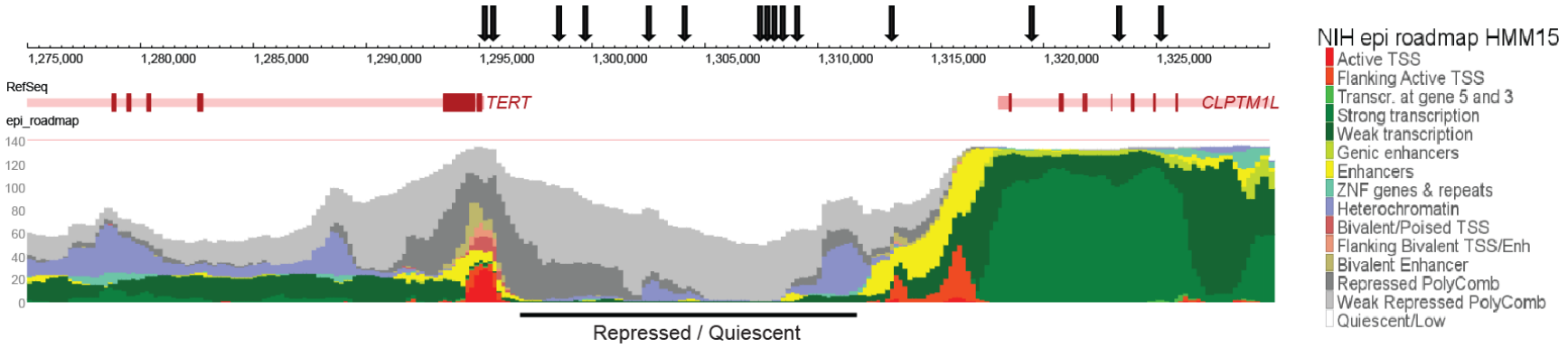
TERT region has gains, losses and inversions in 23% of high stage NB

TERT expression affected



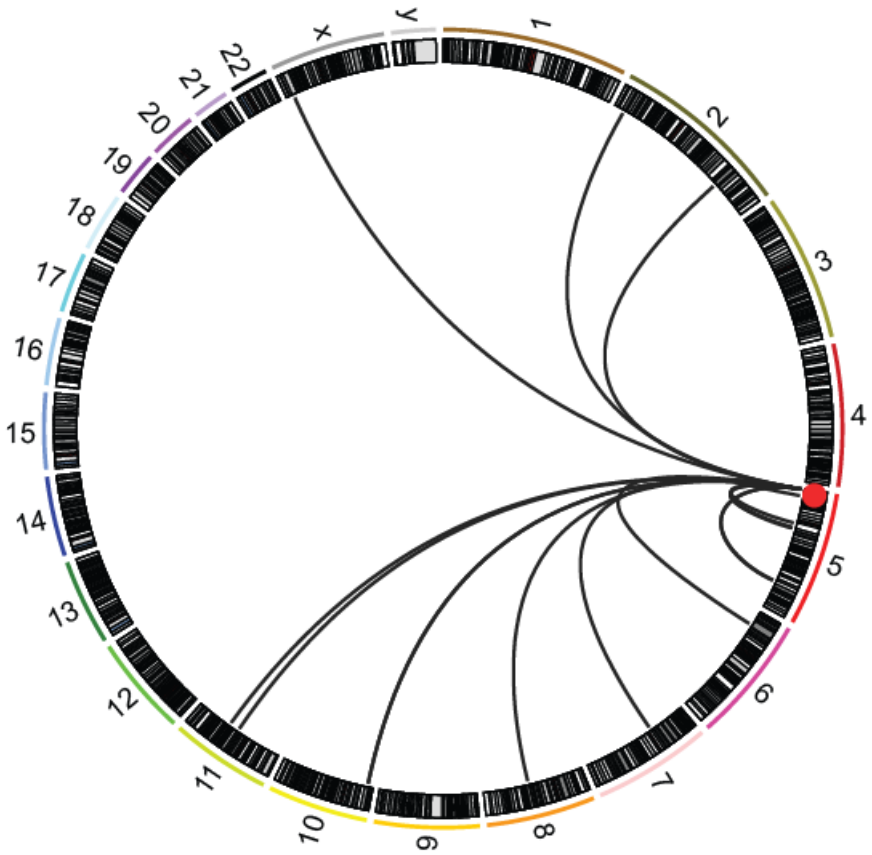
Valentijn & Koster et al 2015 Nat. Genet

TERT upstream region mostly quiescent / repressed



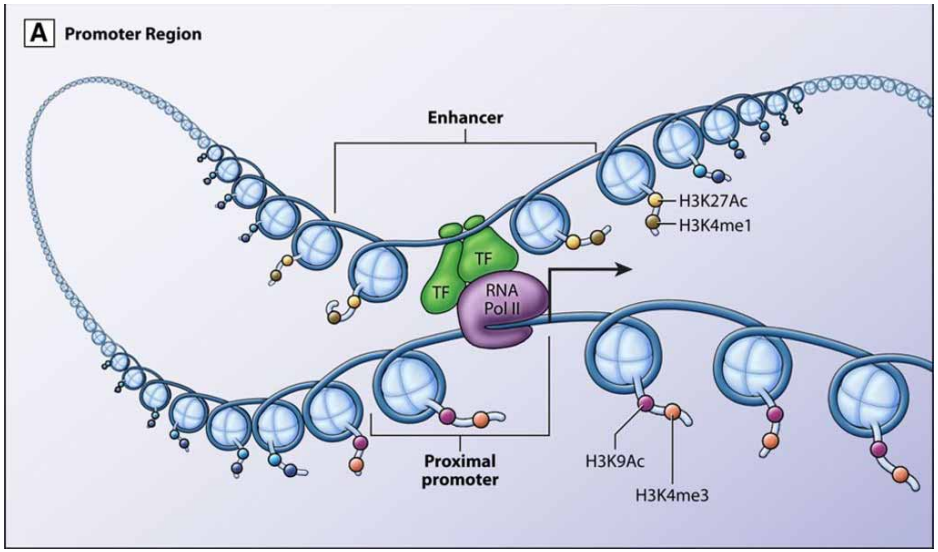
Valentijn & Koster et al 2015 Nat. Genet

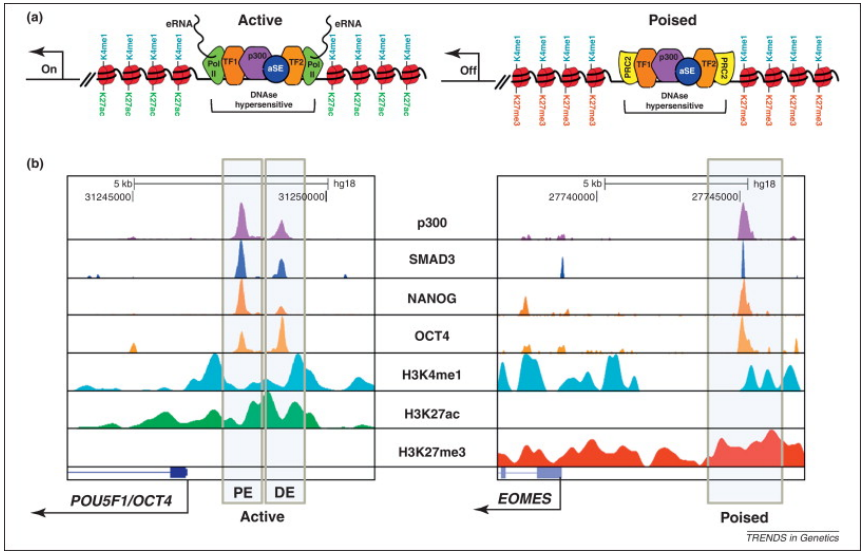
TERT rearrangements have many different translocation partners



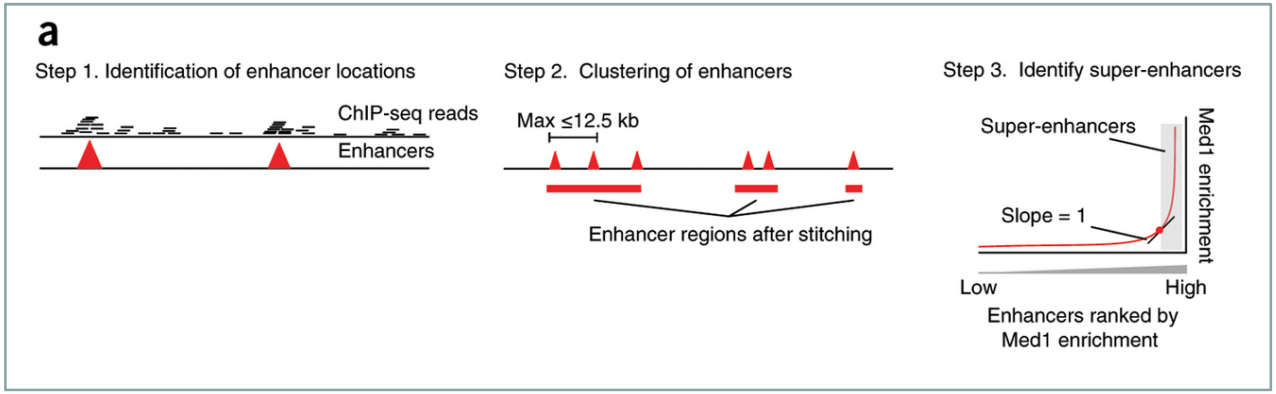
Valentijn & Koster et al 2015 Nat. Genet

Enhancers



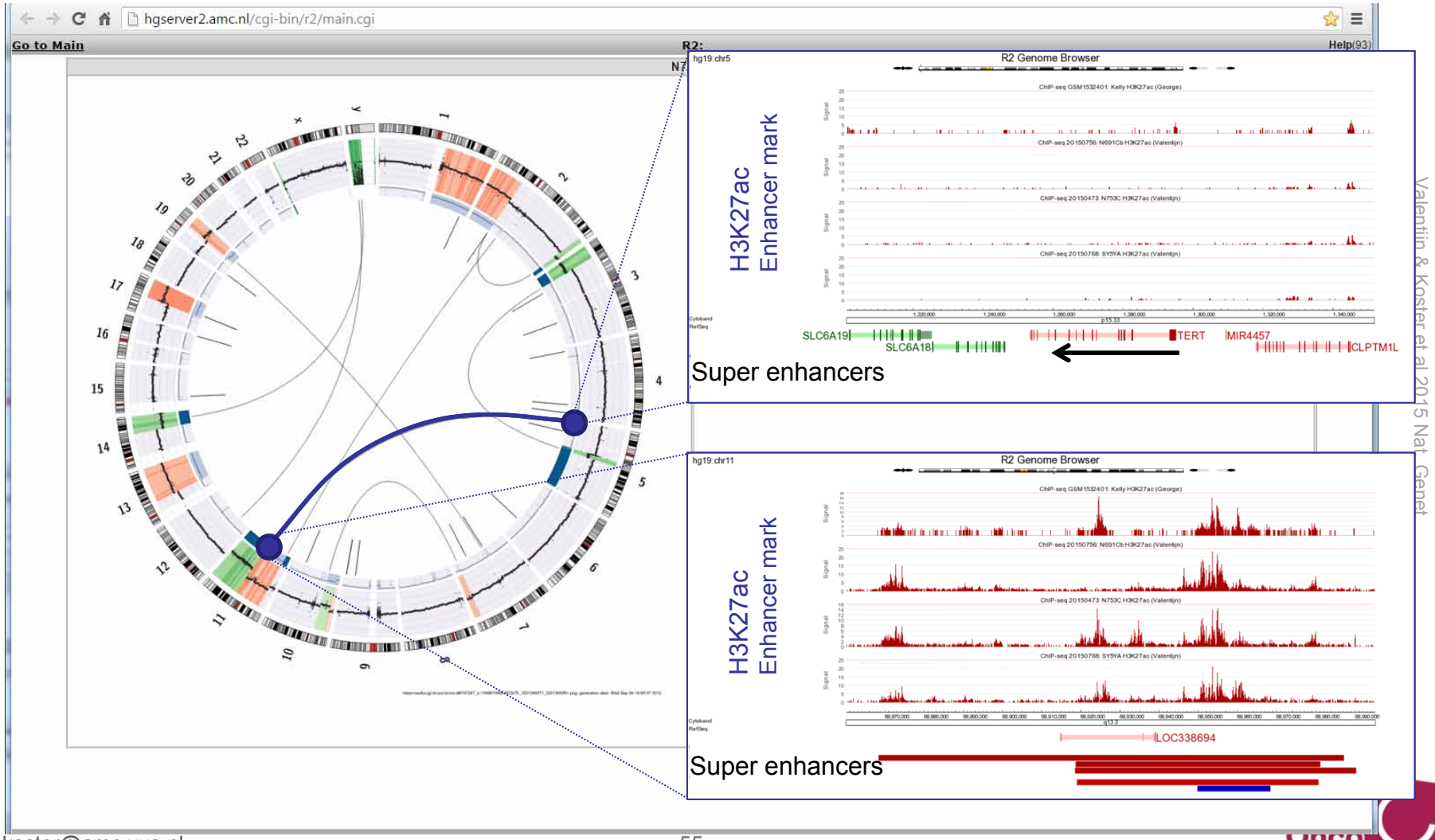


Active enhancers are marked by H3K27Ac

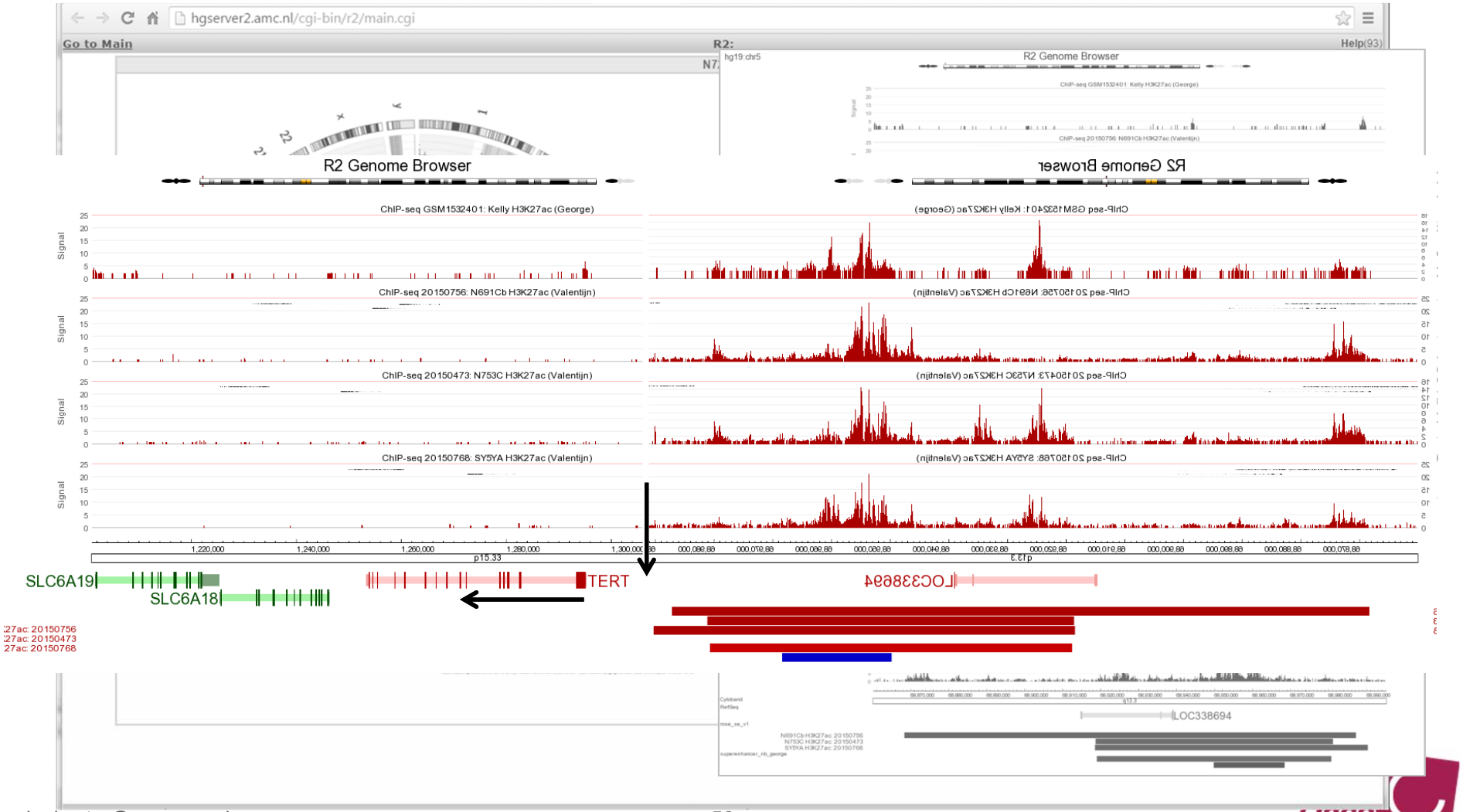


Super Enhancers are the most 'active / enriched' enhancers

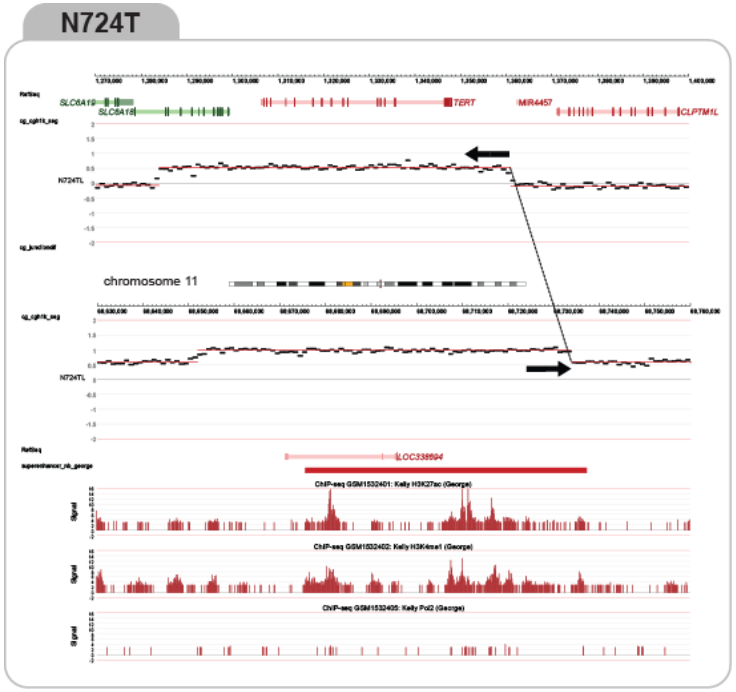
TERT translocates to super-enhancers

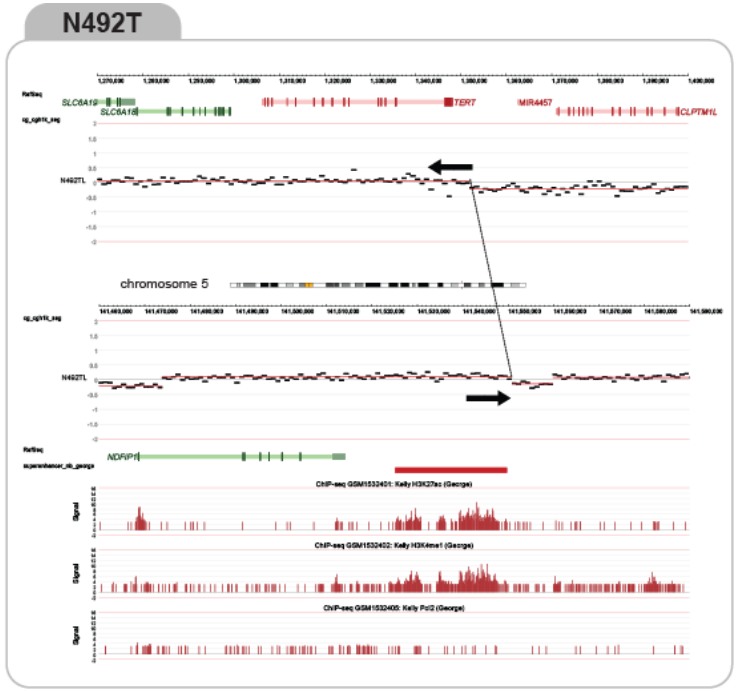


TERT translocates to super-enhancers



TERT translocates to super-enhancers



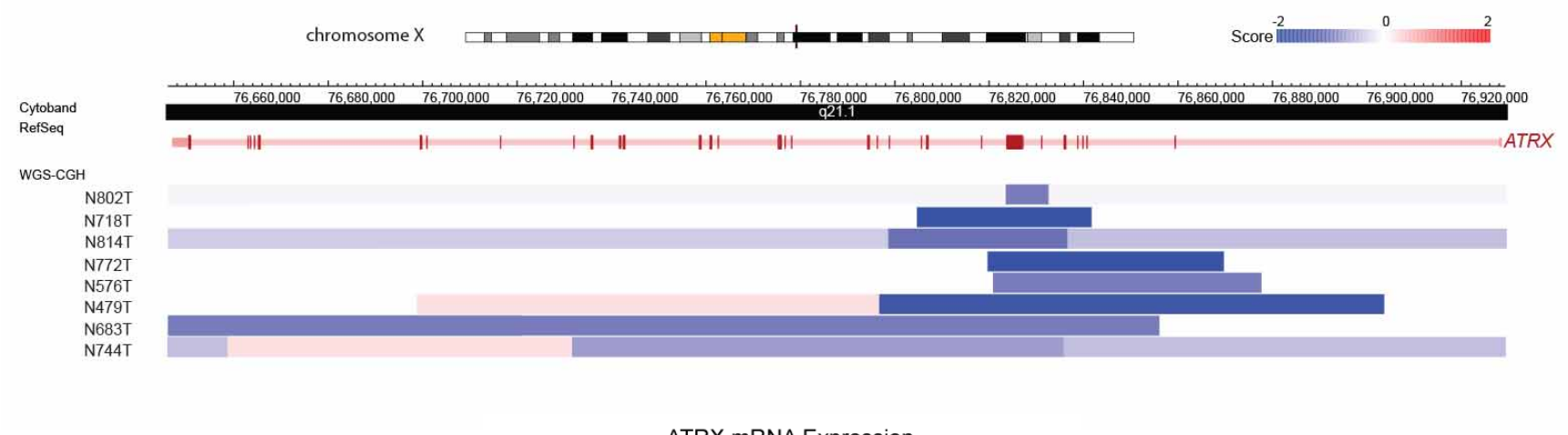


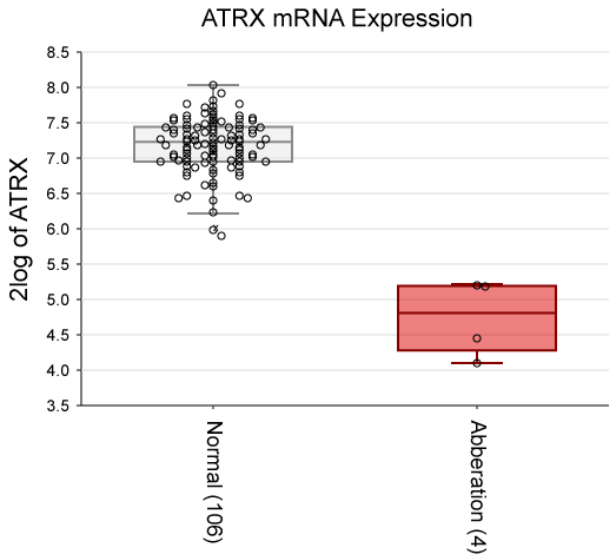
	r100k	r250k	r500k	r750k	r1000k
p count>=5	0.00034				
p count>=6		0.0011			
p count>=7			0.00306		
p count>=9				0.00045	0.00234

100,000 iterations of random breakpoints

Valentijn & Koster et al 2015 Nat. Genet

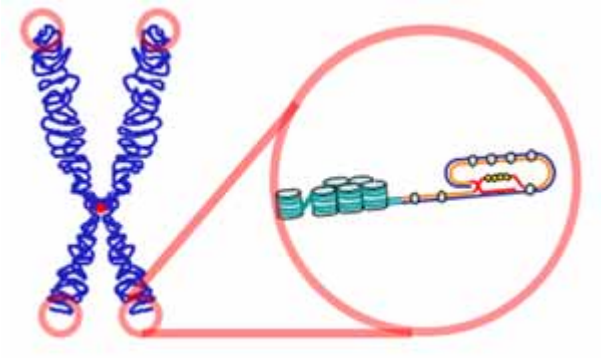
ATRX impairment => ALT





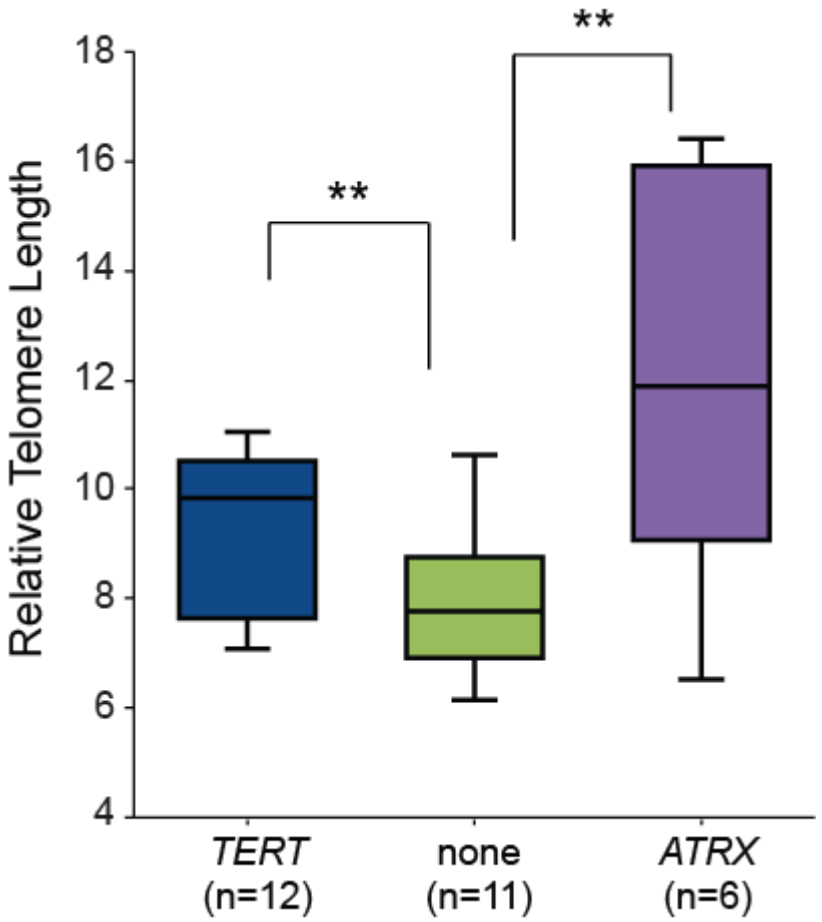
Valentijn & Koster et al 2015 Nat. Genet

Telomeres length NGS



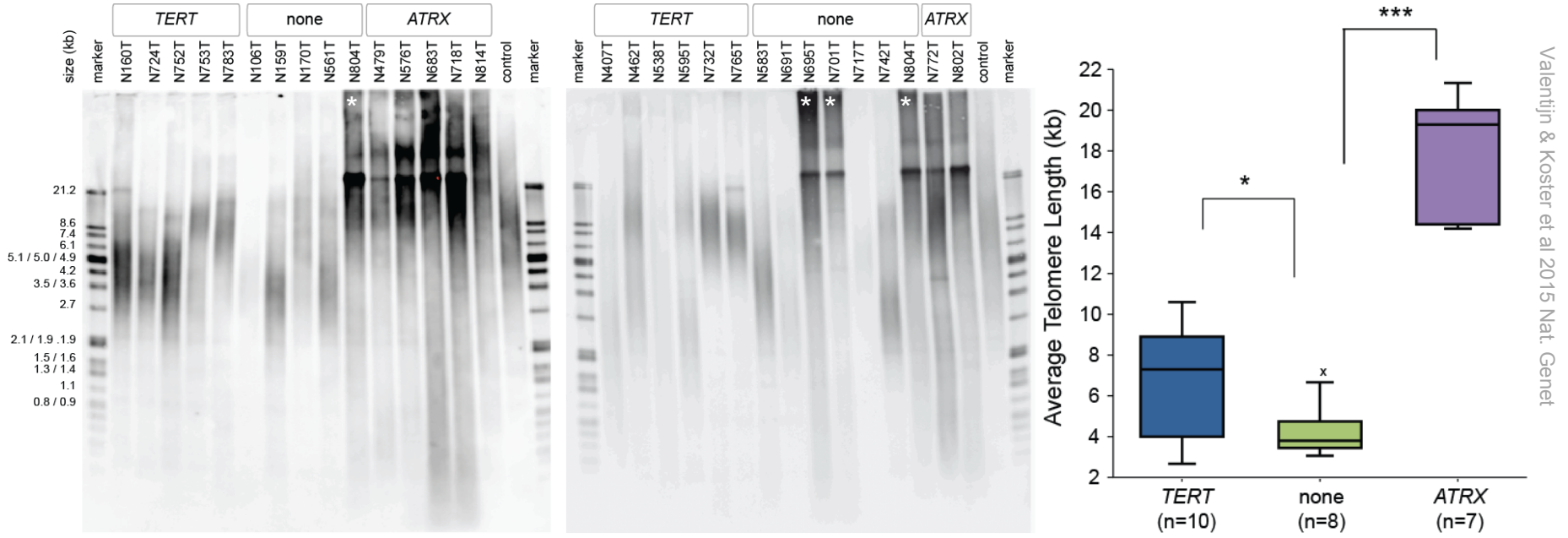
Telomeres are $(TTAGGG)_n$

Search for reads containing multiple instances (>=4) of this sequence and relate to total reads



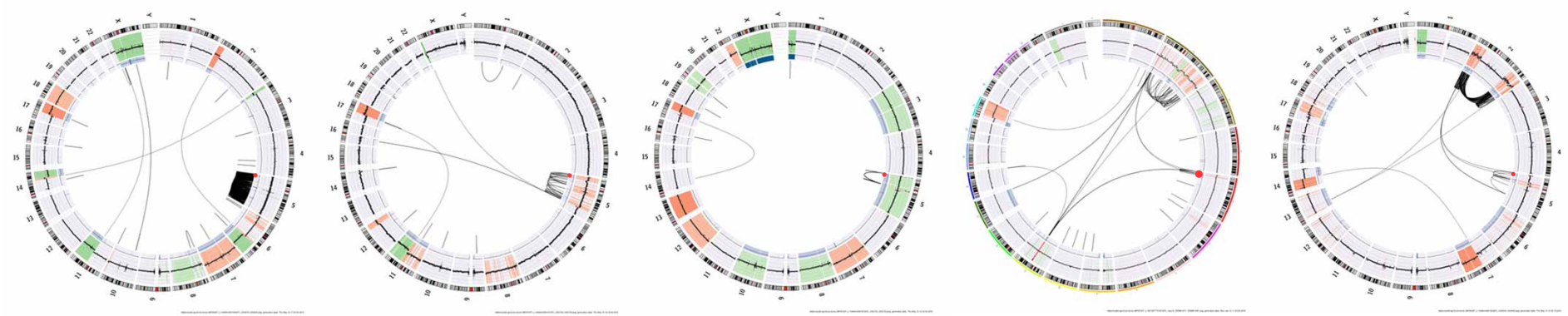
Valentijn & Koster et al 2015 Nat. Genet

Telomere length TRF analysis



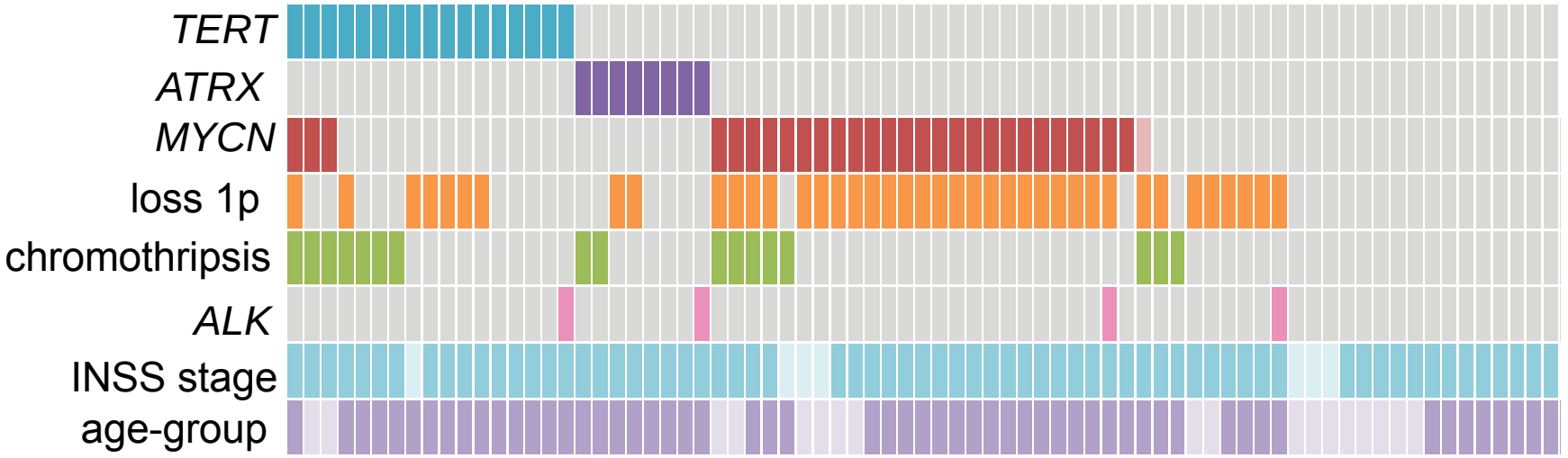
Chromothripsis

- 5 chromothripsis cases directly affect TERT



Genet

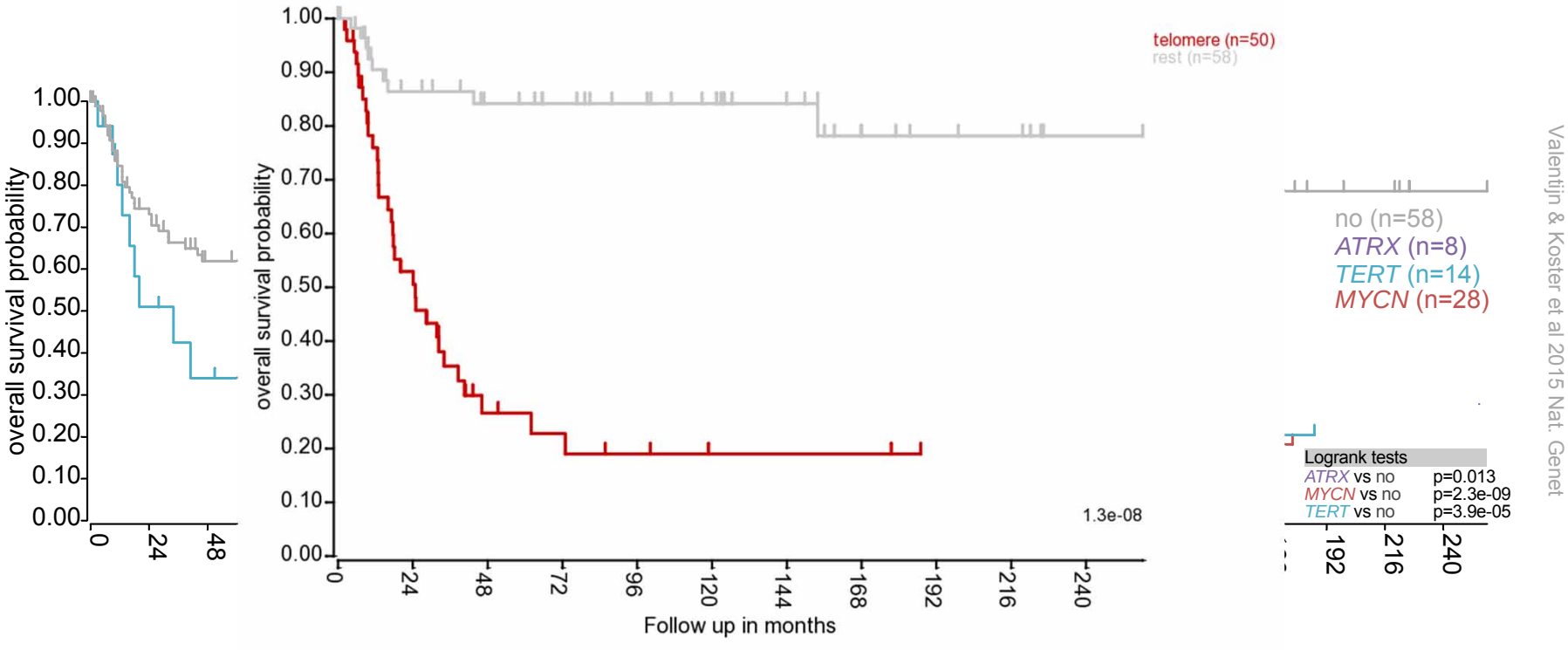
Clinical / Molecular association



TERT rearrangement
ATRX inactivation
MYCN amplification
MYC amplification
chromosome 1p loss
chromothripsis
ALK mutation
wt
stage 4
stage 3
> 18 months
<= 18 months

Valentijn & Koster et al 2015 Nat. Genet

Clinical / Molecular association



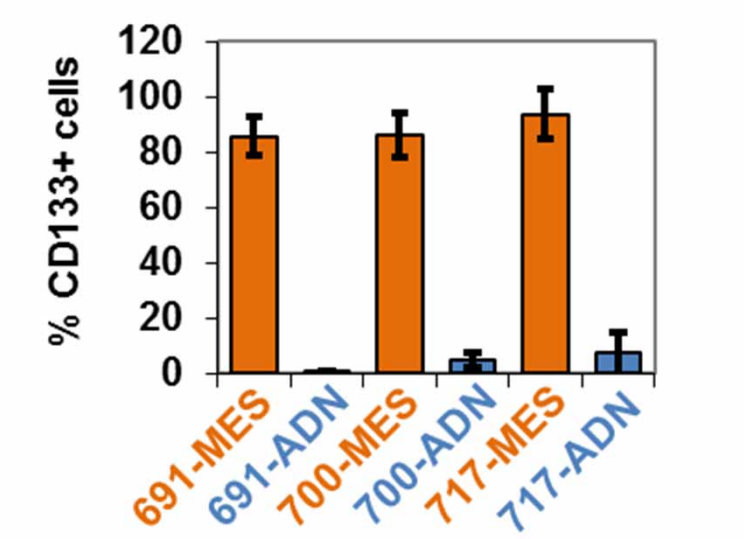
Valentijn & Koster et al 2015 Nat. Genet

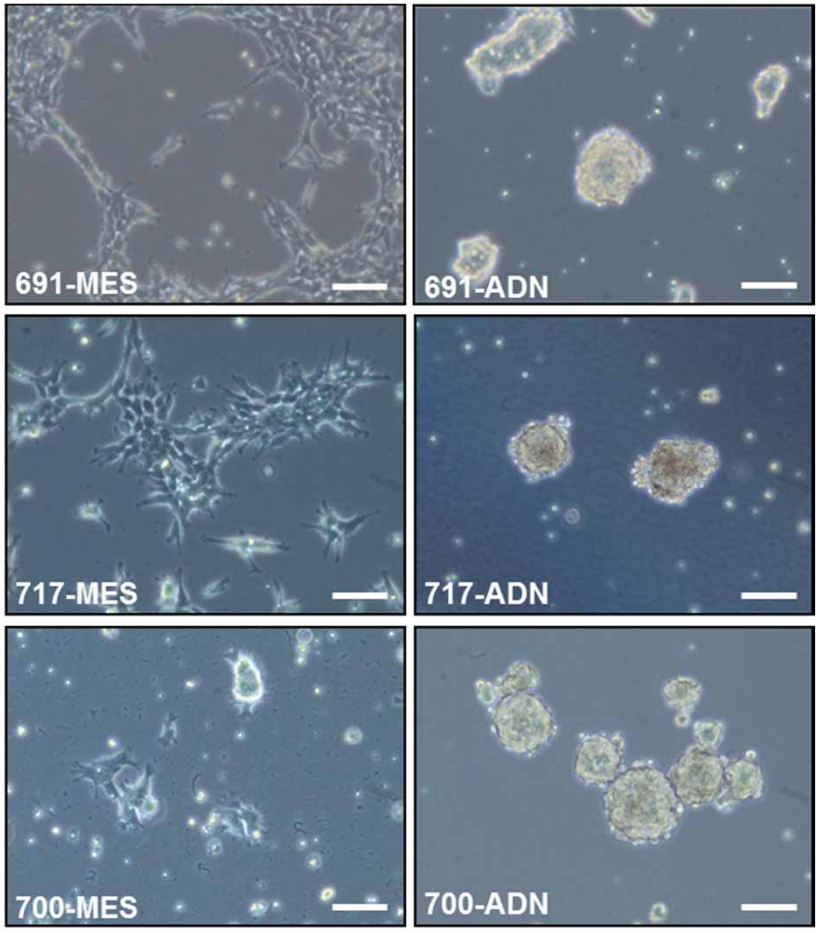
Take home messages on TERT

- TERT region is frequently affected by structural variations in high stage Nb
  - Which leads to upregulation of TERT expression
  - Potentially through enhancer hijacking
  - Is associated with longer telomeres
- TERT affected patients show adverse prognosis
- Subgroup of Nb may be a telomere driven disease
- Pharmacological inhibition of *TERT/ALT* should be explored for this group
  - ATR inhib. for ALT in osteosarcoma (Flynn *et al.* Science 2015)
  - Telomerase inhib. In neuroblastoma (Kremer *et al.* , ANR2016)

Neuroblastoma sub-clones

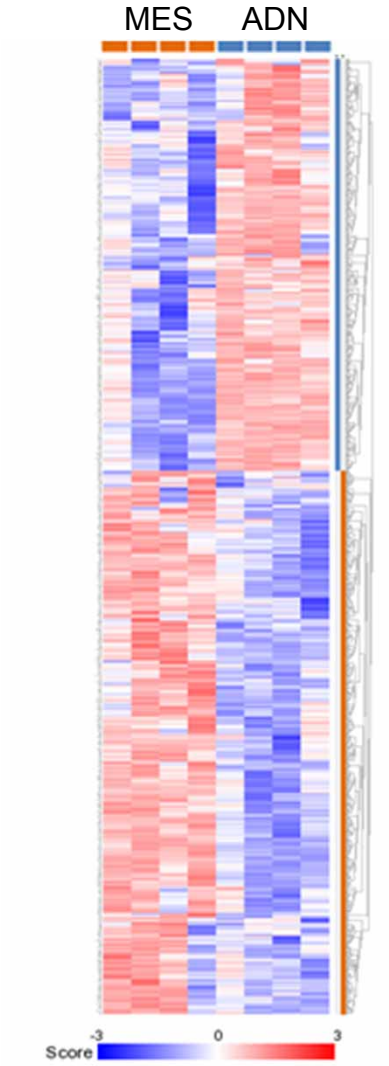
- Patient derived cell lines
  - Multiple lines from same patient
  - Different phenotypes
    - ‘Stem cell’ marker CD133
    - Mesenchymal properties

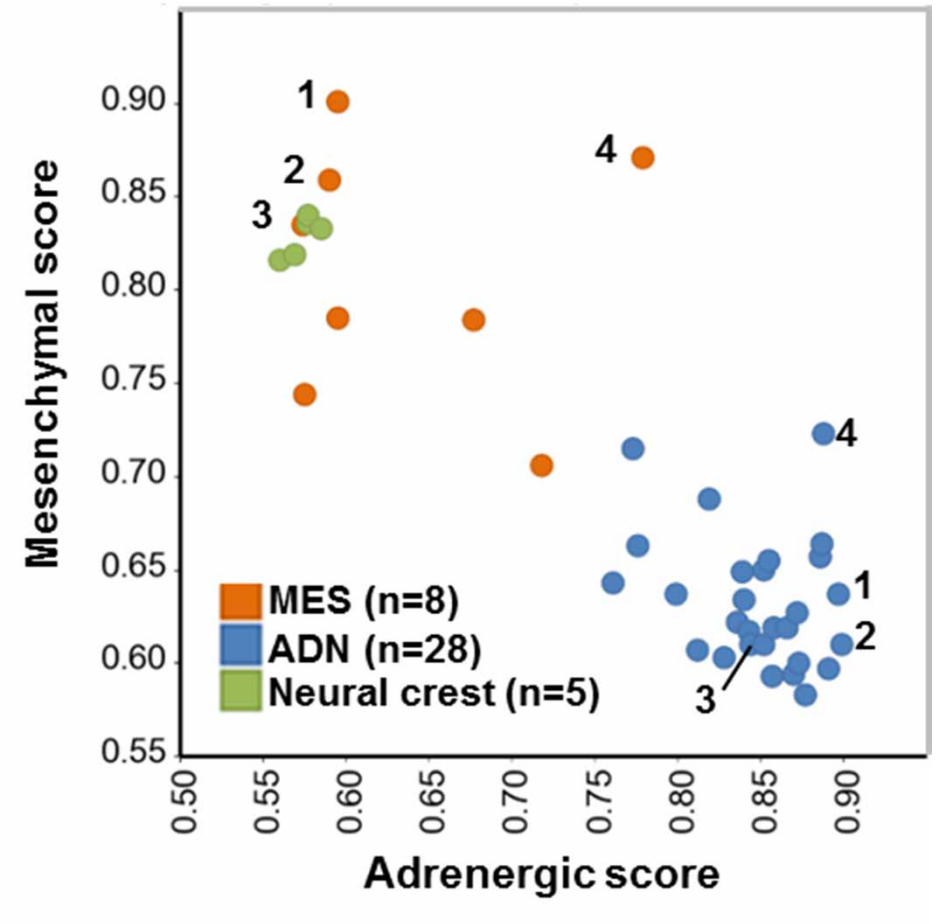




van Groningen & Koster et al 2017 Nat. Genet (in revision)

Pairs segregate along a MES/ADN axis





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Pairs can interconvert into one another

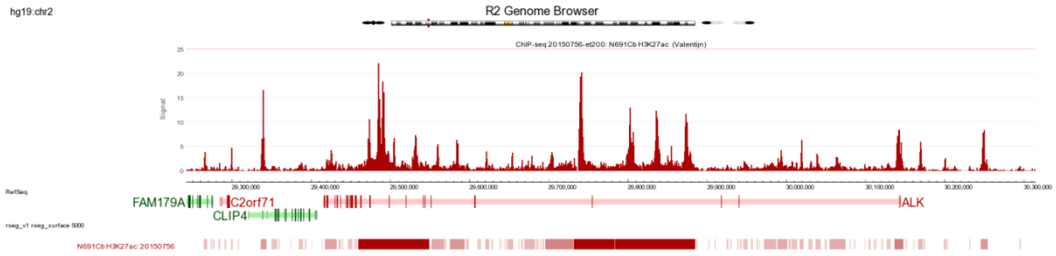
- Pairs are vastly different on mRNA level
- N700 parental line is mixed (MES/ADN)
  - FACS sorting (CD133) for one always results in mixture containing a small sub population of the other
  - Even after single cell sorting
  - In mice, also tumors from CD133^{-} cells were always heterogeneous

- Interconversion occurs

- Incompatible with genetic differences
- Enhancer driven?

Enhancer Landscape

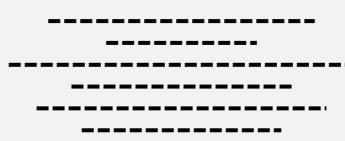
- Assessed enhancer landscape in 9 samples
  - 4 X MES
  - 5 X ADN



E.g. 4 sets of ROSE SE regions

Overlay all sets and determine regions

Align regions SE>= 2 cell lines



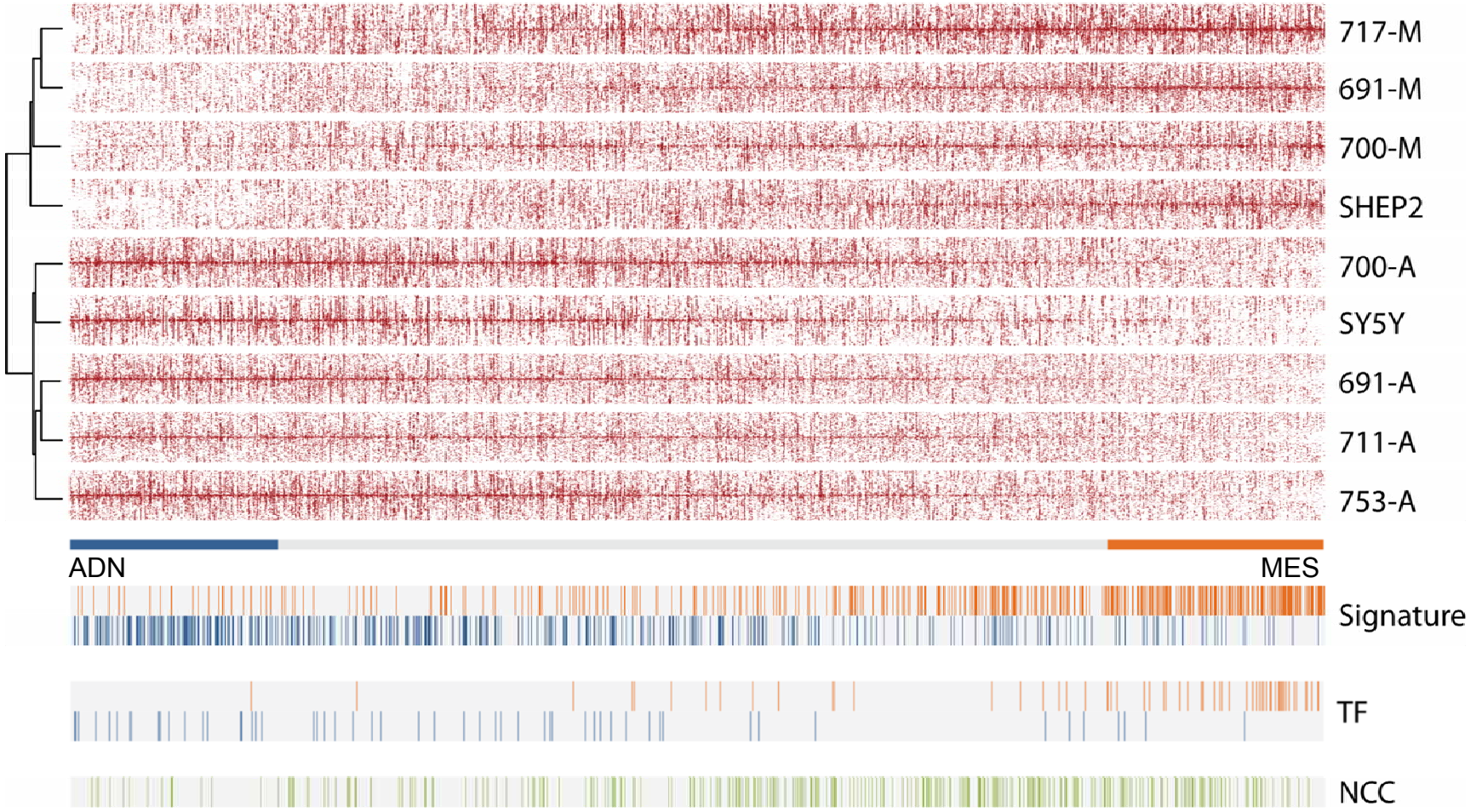
Cluster Profiles

Exclude transcription induced signals
Merge to point estimates
Cluster on samples
Order on diff. Signals (MES/ADN)

Prepare for visualization

Replace point estimates by SE peak profiles (+/- 1mb)

SuperEnhancer Landscape MES/ADN



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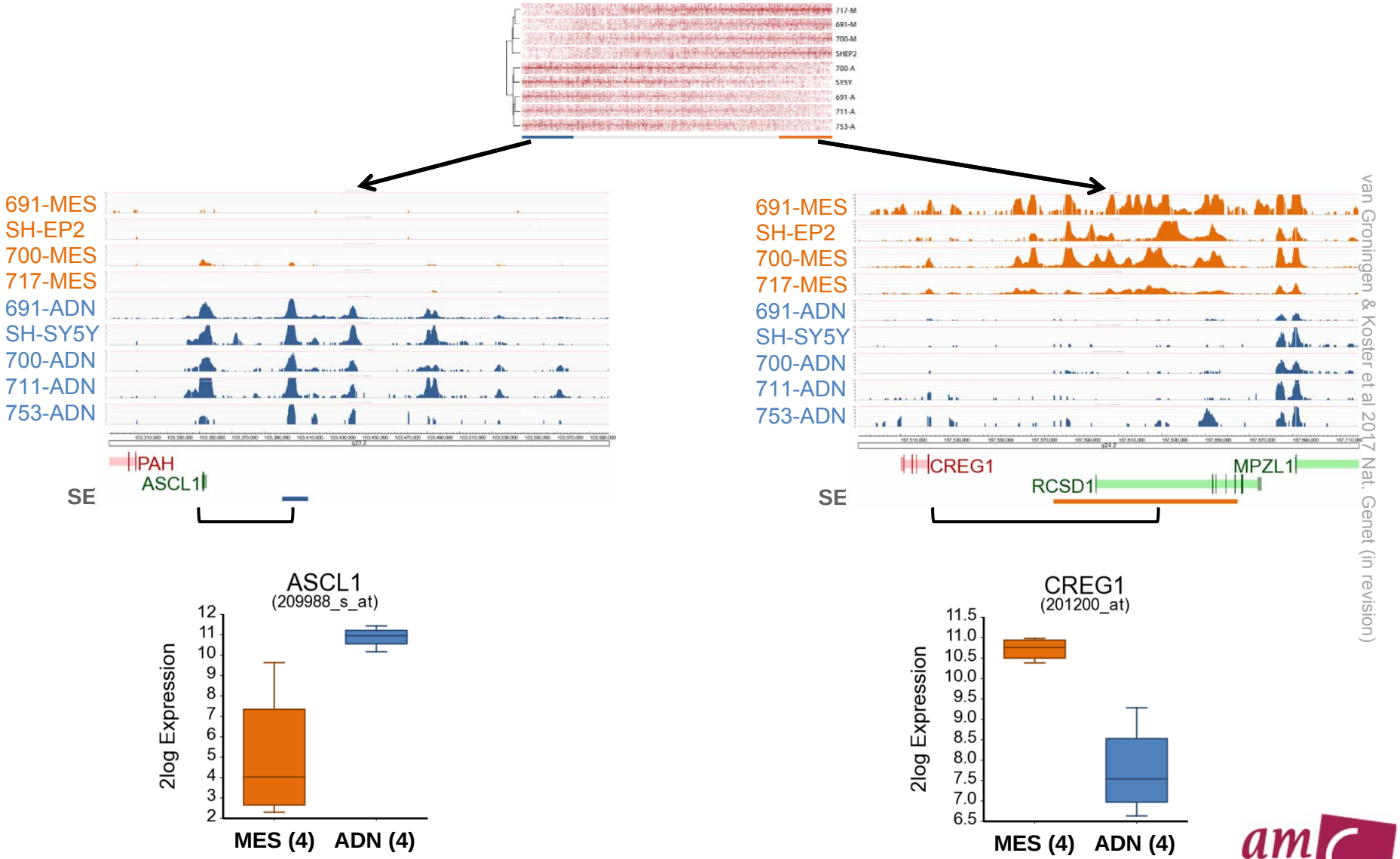
SuperEnhancer Landscape MES/ADN



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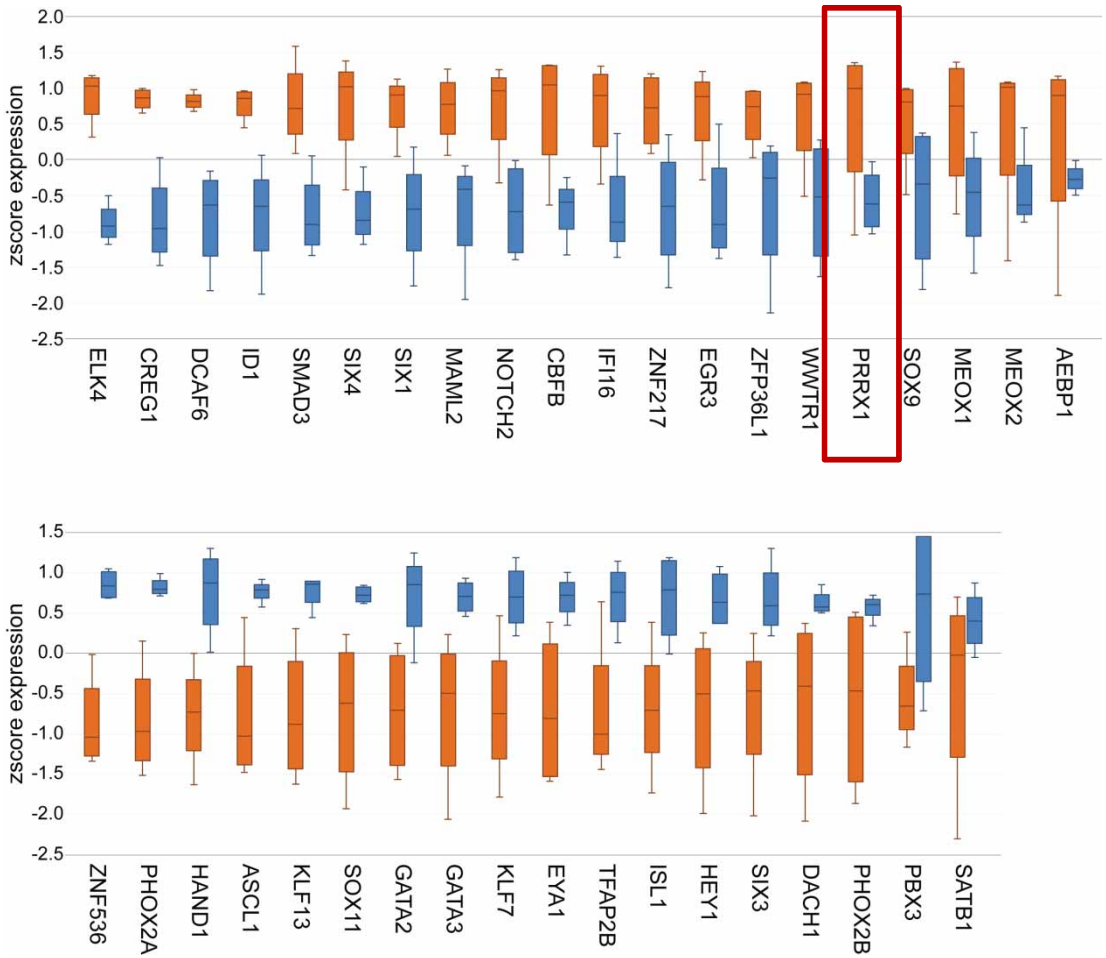
Transcription Factors amongst most differentially expressed genes



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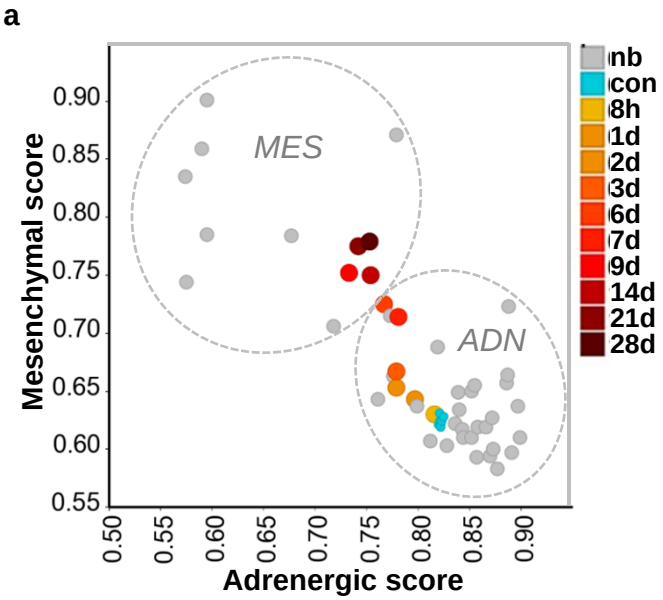
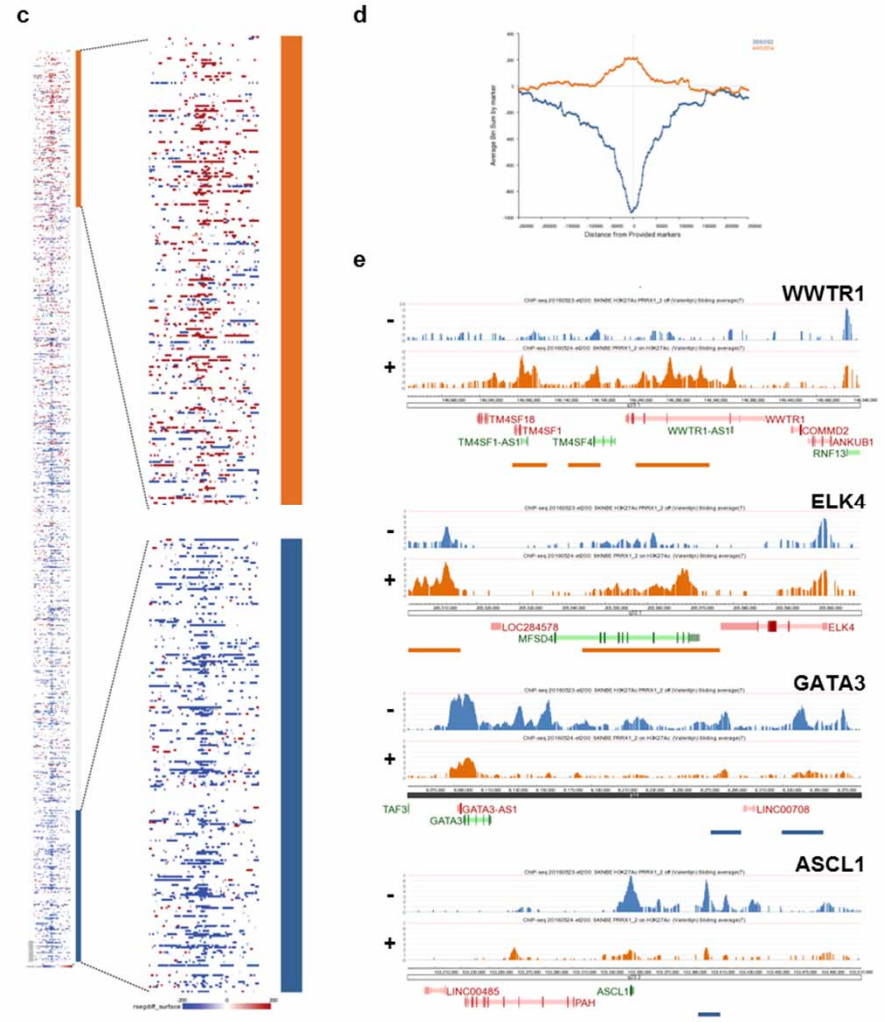
Introduction of PRRX1 into ADN cell line leads to MES transformation

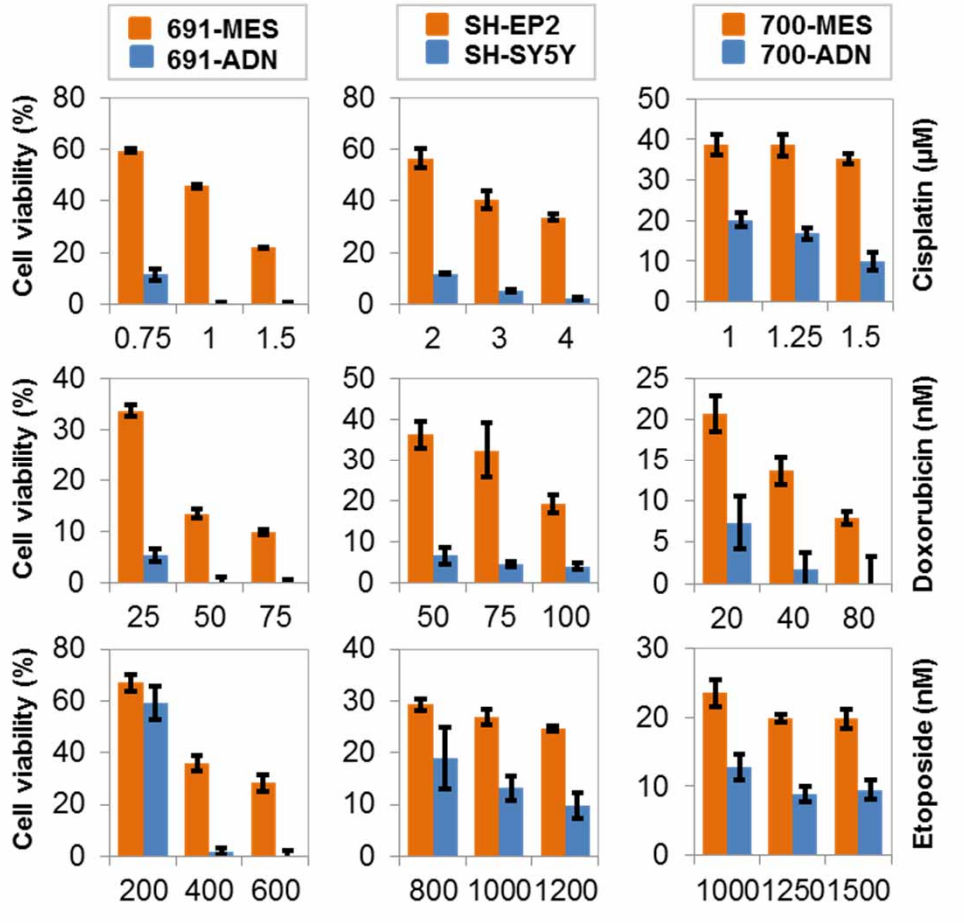
SE status after 12 days (PRRX1 on/off)



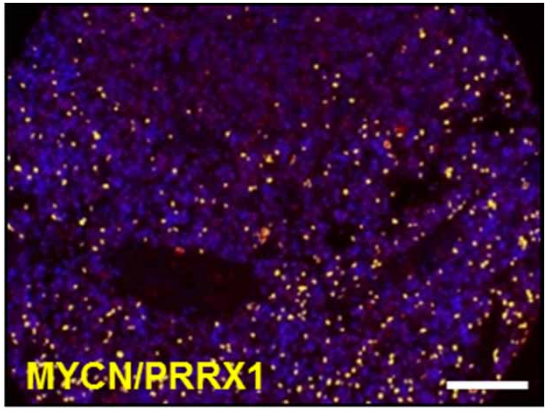


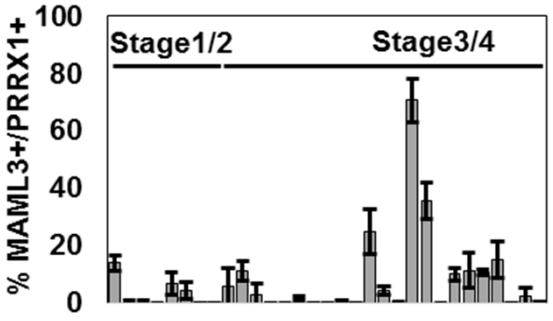
van Groningen & Koster et al 2017 Nat. Genet (in revision)

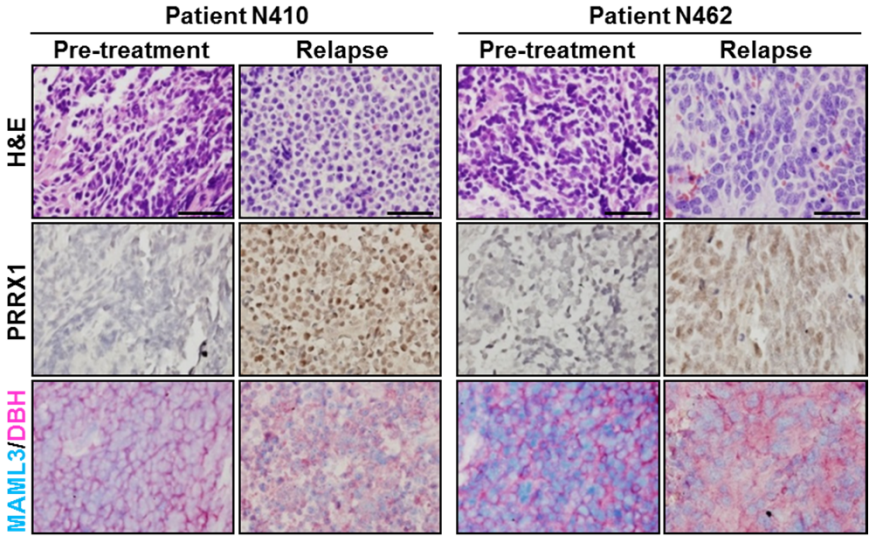
MES cells are more resistent to therapy

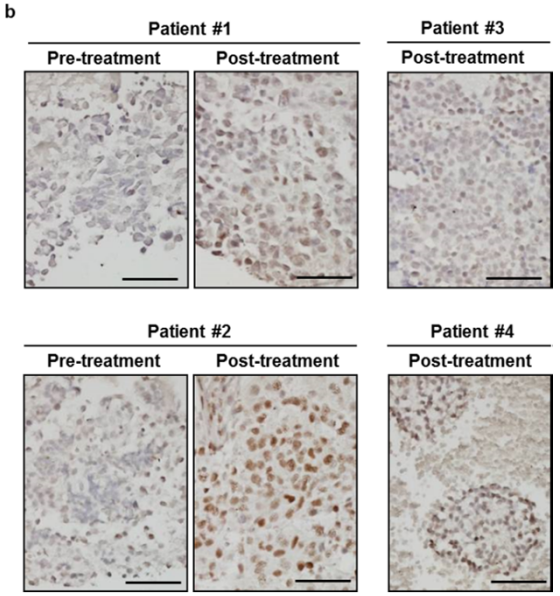


MES cells do exist *in-vivo* and may be enriched upon treatment









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Take home messages subclones

- Neuroblastoma contains at least 2 sub-types of cells
  - ADN / MES
- Sub-types can interconvert
  - Associates with different SE landscapes and gene expression profiles
- PRRX1 can impose a MES switch in ADN cells
  - And SE landscape
- MES cells more resistent to therapy
  - Enriched upon treatment / and relapse
- Could have profound implications for patient treatment

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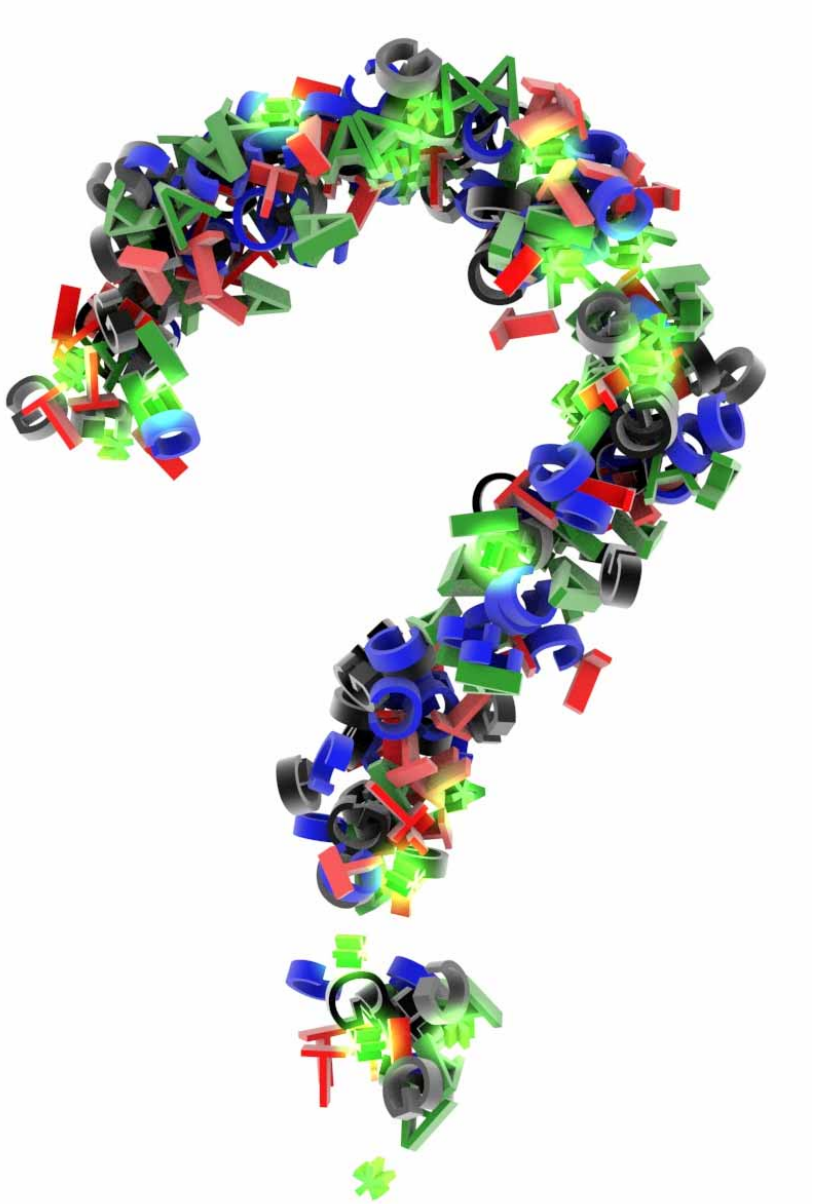
Acknowledgements

- Oncogenomics Amsterdam (AMC)
  - Bioinformatics
    - Jan Koster
    - Danny Zwijnenburg
  - Wet-lab biology
    - Jan Molenaar
    - Marli Ebus
    - Ida van der Ploeg
    - Linda Valentijn
    - Ellen Westerhout
    - Peter van Sluis
    - Johan van Nes
    - Tim van Groningen
    - Thomas Eleveld
  - Rogier Versteeg
- BGI/Complete Genomics
  - Rick Tearle
  - Greg Tyrelle



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