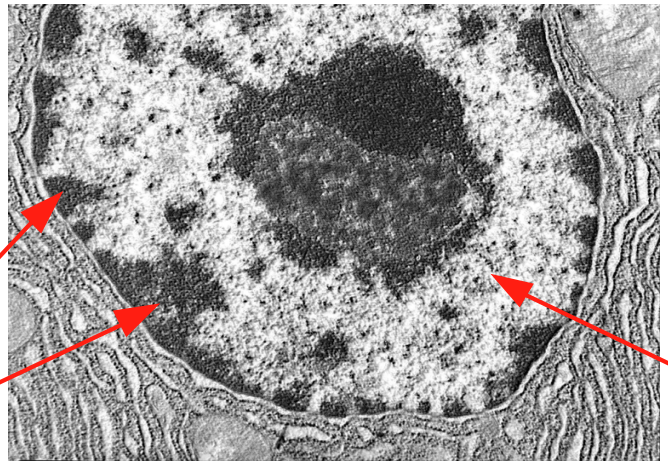
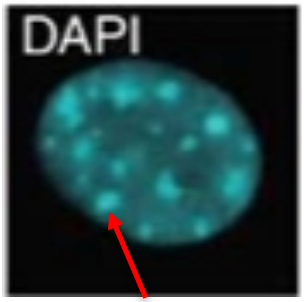
HOW TO STUDY EPIGENETIC MODIFICATIONS

-- STRATEGIES USING SUV39H1 AS A HALLMARK MODEL FOR EPIGENTIC REGUALTION --

Chromatin comes in different flavors

Different types of chromatin





Chromocenter
(aggregates of centromeres
= constitutive heterochromatin)

heterochromatin

euchromatin
(and facultative heterochromatin)

Constitutive heterochromatin:

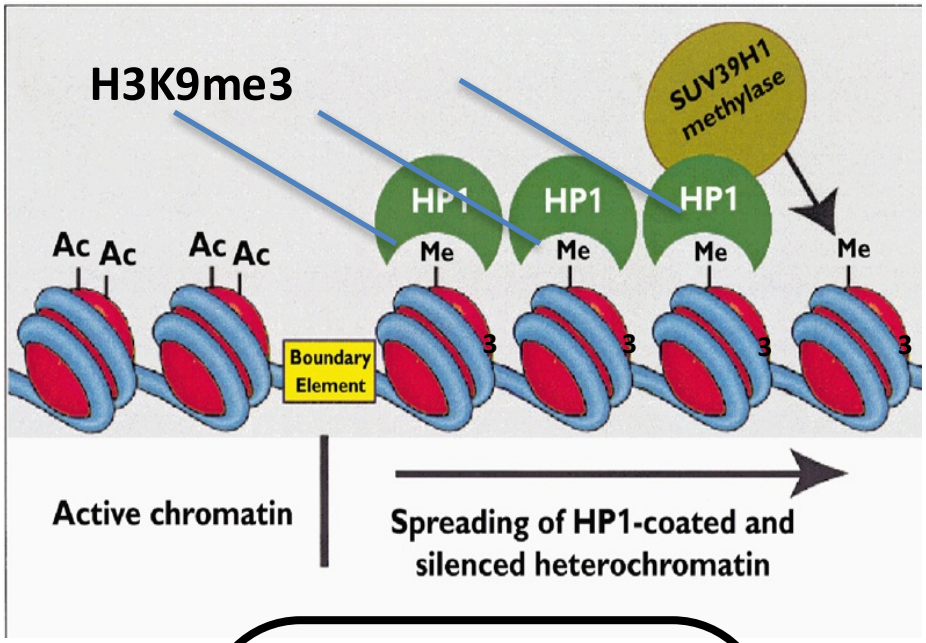
- constitute ~ 10% of nuclear DNA; telomeres, centromeres, and a considerable fraction of repetitive sequences
- highly compacted, replicates late in S phase, (transcriptionally inert)

Euchromatin + facultative heterochromatin:

- constitute ~ 90% of nuclear DNA
- less condensed, rich in genes, replicates early in S phase
  however,
- only small fraction of euchromatin is transcriptionally active
- the rest is transcriptionally inactive/silenced (but can be activated in certain tissues or developmental stages) → these inactive regions are also known as "facultative heterochromatin"

Post-translational histone modifications can recruit specialized proteins

Example: SUV39H1 and HP1 form heterochromatin at centromeric and telomeric Heterochromatin in flies and vertebrates and SAHFs



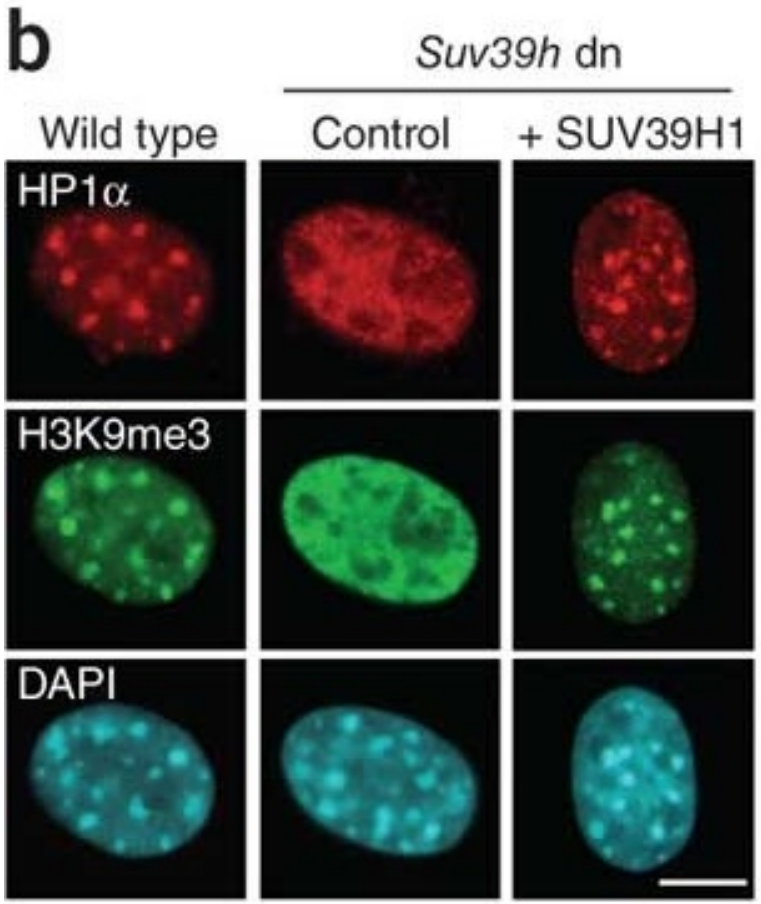
Suv39h1/2 tri-methylate Lysine 9 on histone H3

↓

tri-methylated Lysine 9 on histone H3 recruits HP1

↓

heterochromatin

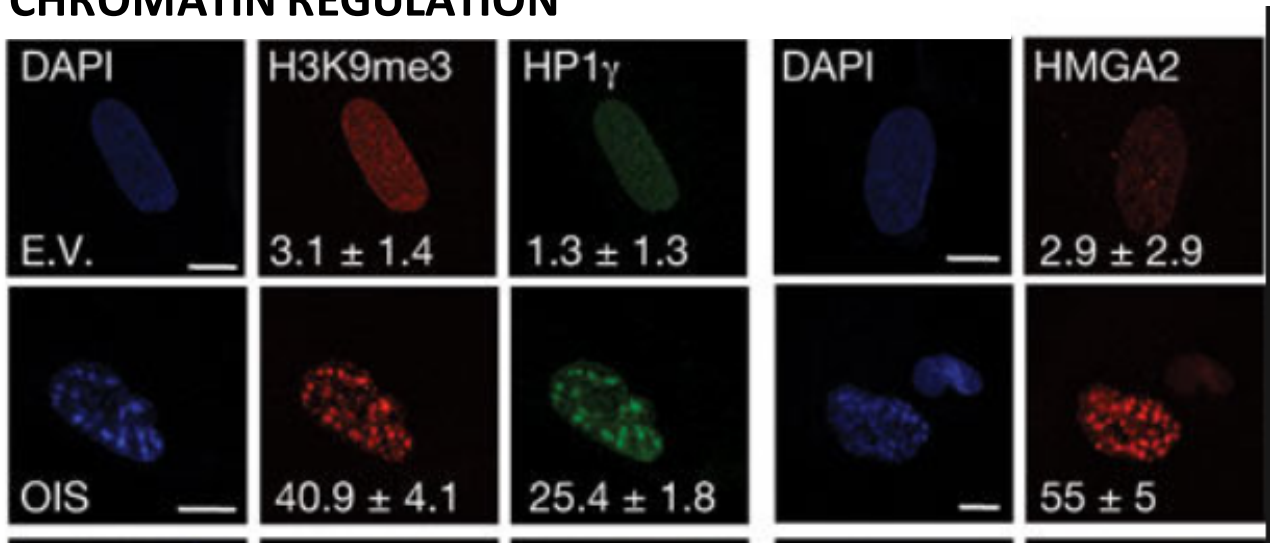


histone modifications can reach high levels in cells and can be visualized by immunofluorescence

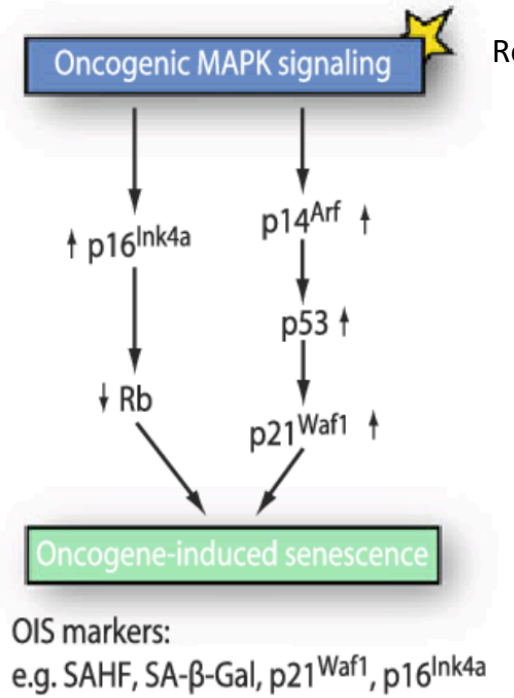
Post-translational histone modifications can recruit specialized proteins

Example: SUV39H1 and HP1 form heterochromatin at centromeric and telomeric Heterochromatin in flies and vertebrates and SAHFs

CELLULAR STIMULUS IS TRANSLATED IN TO CHROMATIN REGULATION



Senescence activated heterochromatin foci (SAHF)



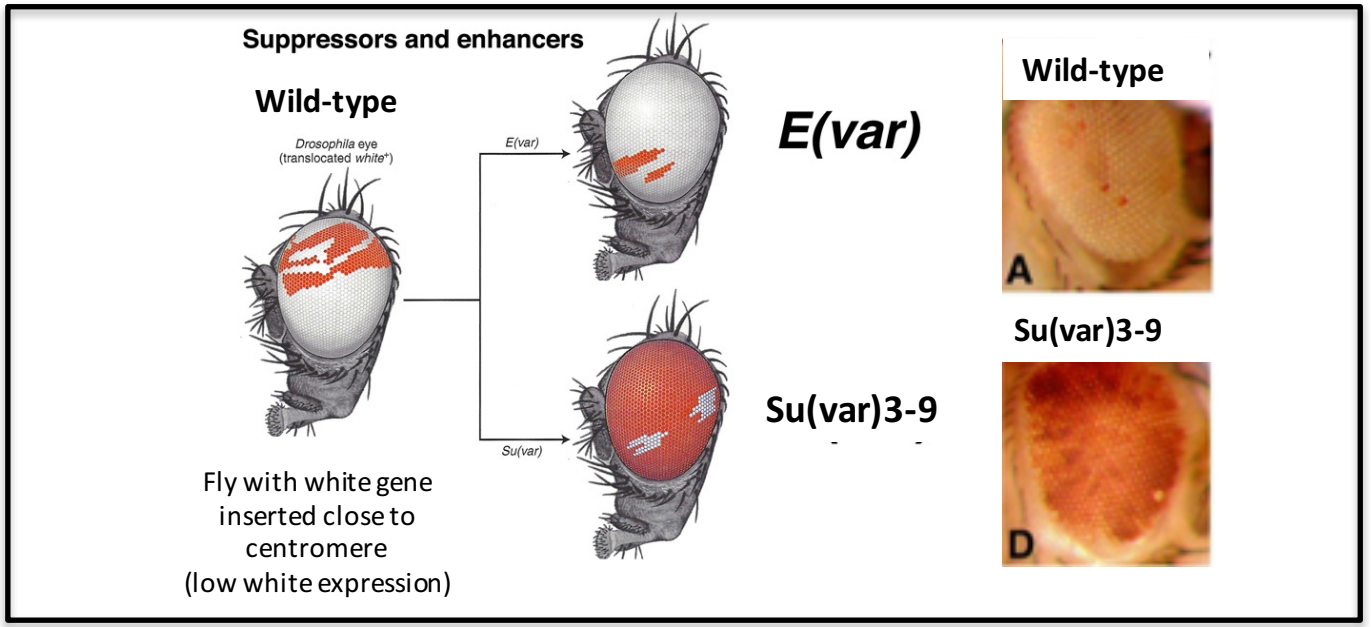
Oncogene induced Replication stress → DNA damage

OIS: Oncogene induced senescence

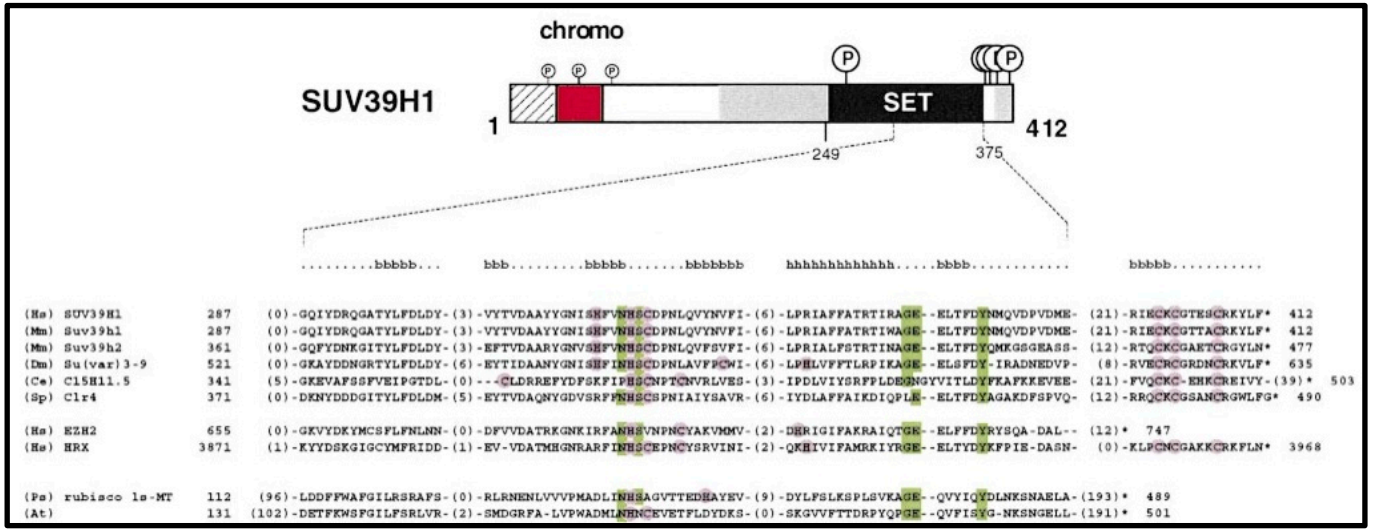
A historical view: how to understand the enzymatic function of an epigenetic writer
Histones can be acetylated – can they be methylated??

Candidate gene:
Human
SUV39H1 and SUV39H2

Evidence 1
Drosphila
Suv(var)3-9
mutant shows
strong PEV



Evidence 2
Suv39h genes
show high sequence
conservation to
a plant gene with
proposed histone-
methyltransferase
activity



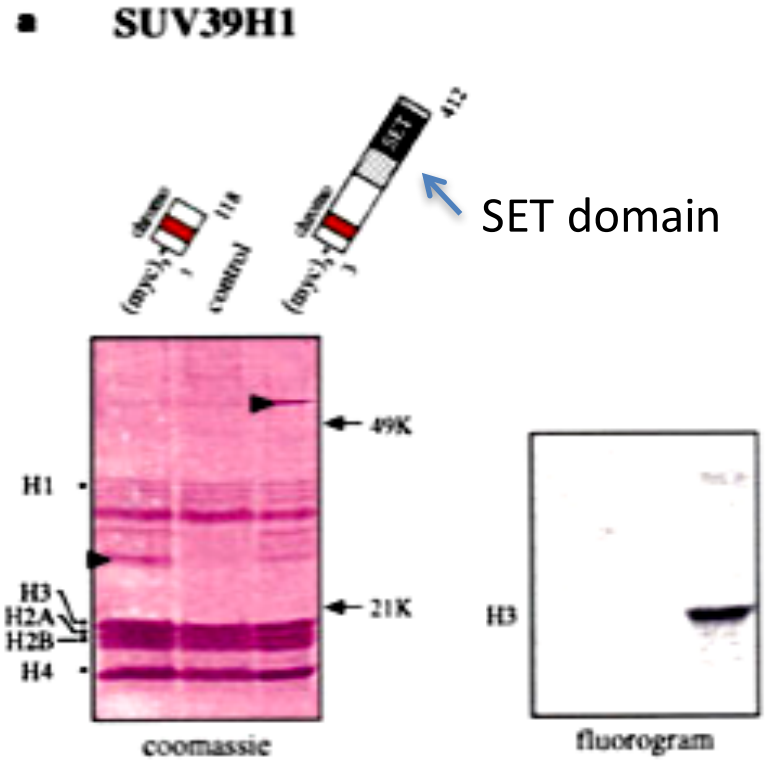
Identification of H3 Lys9 methyltransferase activity

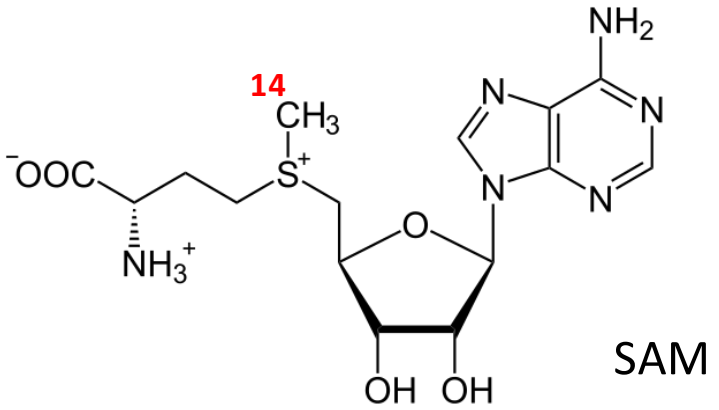
Experiment:

Overexpression of myc-tagged-SUV39H1 in Hela cells

Use an antibody to immunoprecipitate mycSUV39H1 → high concentration of SUV39H1

Incubate Immunopreciptate with purified histones and S-adenosyl-[methyl-^{14}C]-$_L$-methionin as methyl donor





SET – domain is required for histone methyl transferases activity

- The SET domain of the SUV39H1 is required for histone methyltransferase activity and this enzyme methylates H3 at Lys9

Nature. 2001 Mar 1;410(6824):116-20.

Methylation of histone H3 lysine 9 creates a binding site for HP1 proteins.

Lachner M[1], O'Carroll D, Rea S, Mechtler K, Jenuwein T.

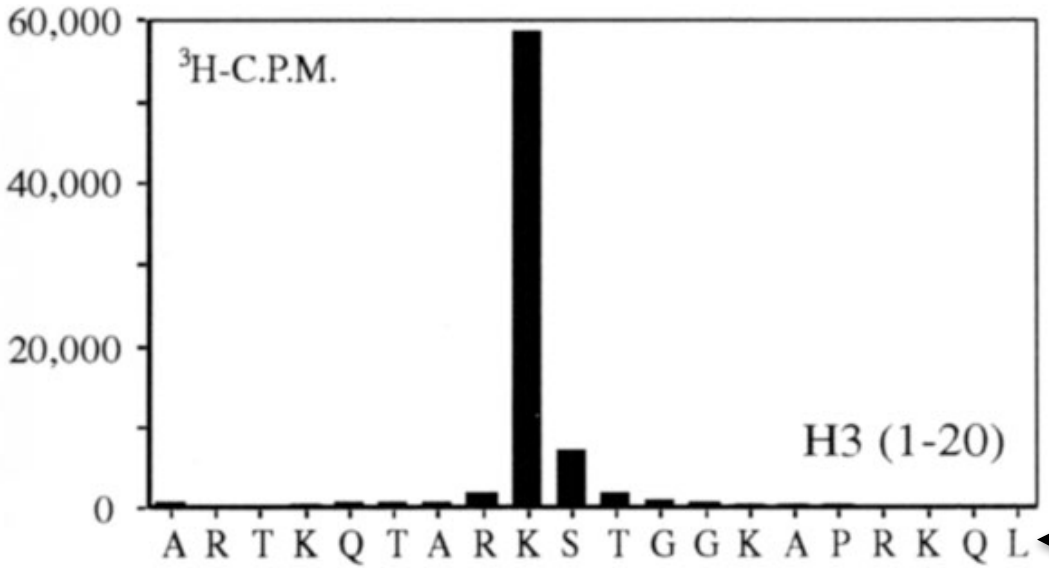
Identification of H3 Lys9 methyltransferase activity

Experiment:

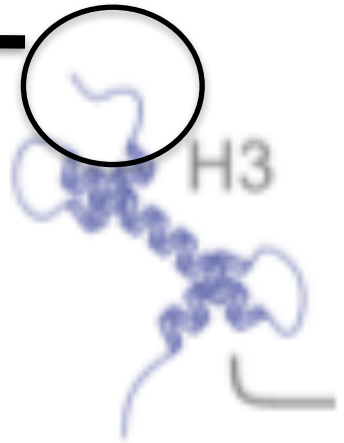
Purify histone H3 by reverse-phase-liquid chromatography after HMTassay

Sequence histone H3 and quantify the amount of radioactivity per ammino acid

Suv39H1 methylates histone H3 at lysine 9

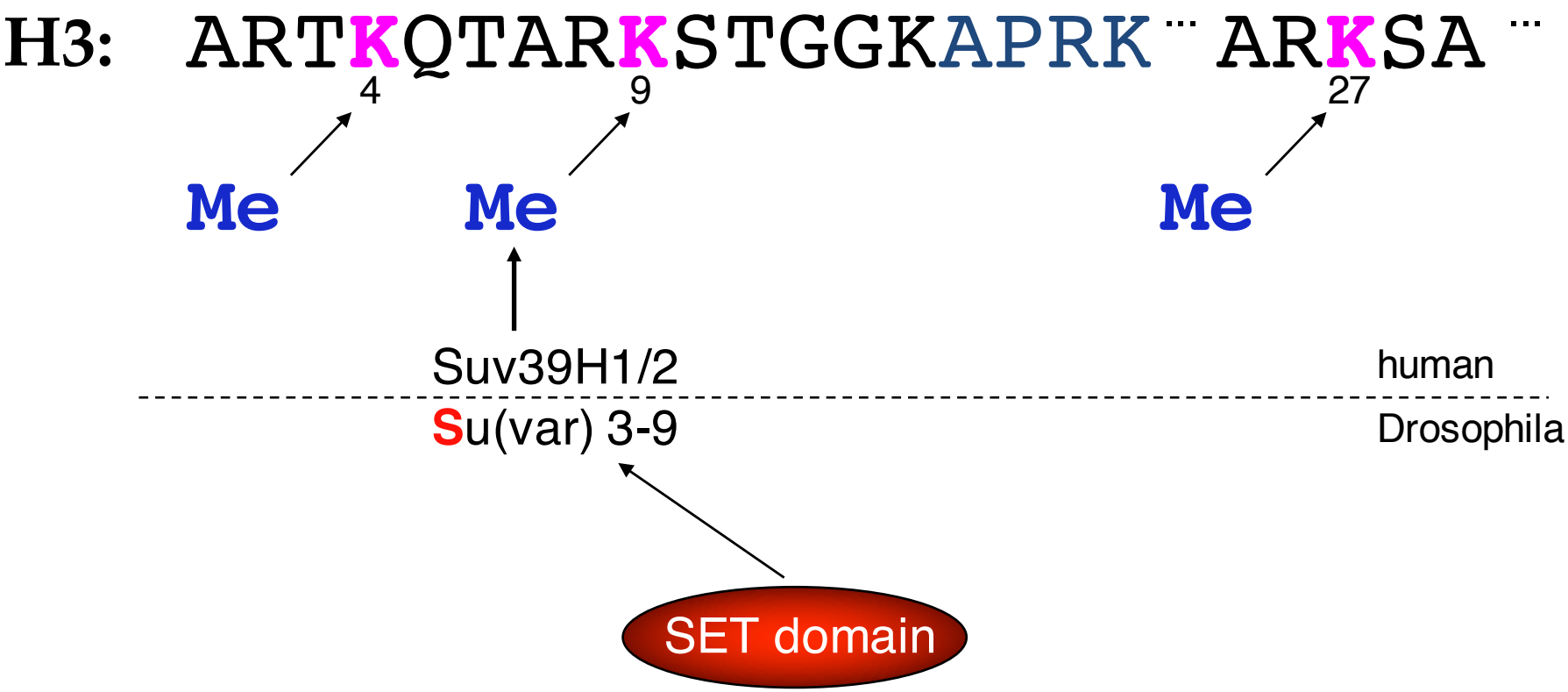


Pos. 1 **Histone H3 aminoacid sequence** Pos. 20



Identification of other Histone H3 Lys9 methyltransferases

- The SET domain is the conserved catalytic core of histone methyltransferases
- The histone H3 tail has 3 sites for methylation at lysines



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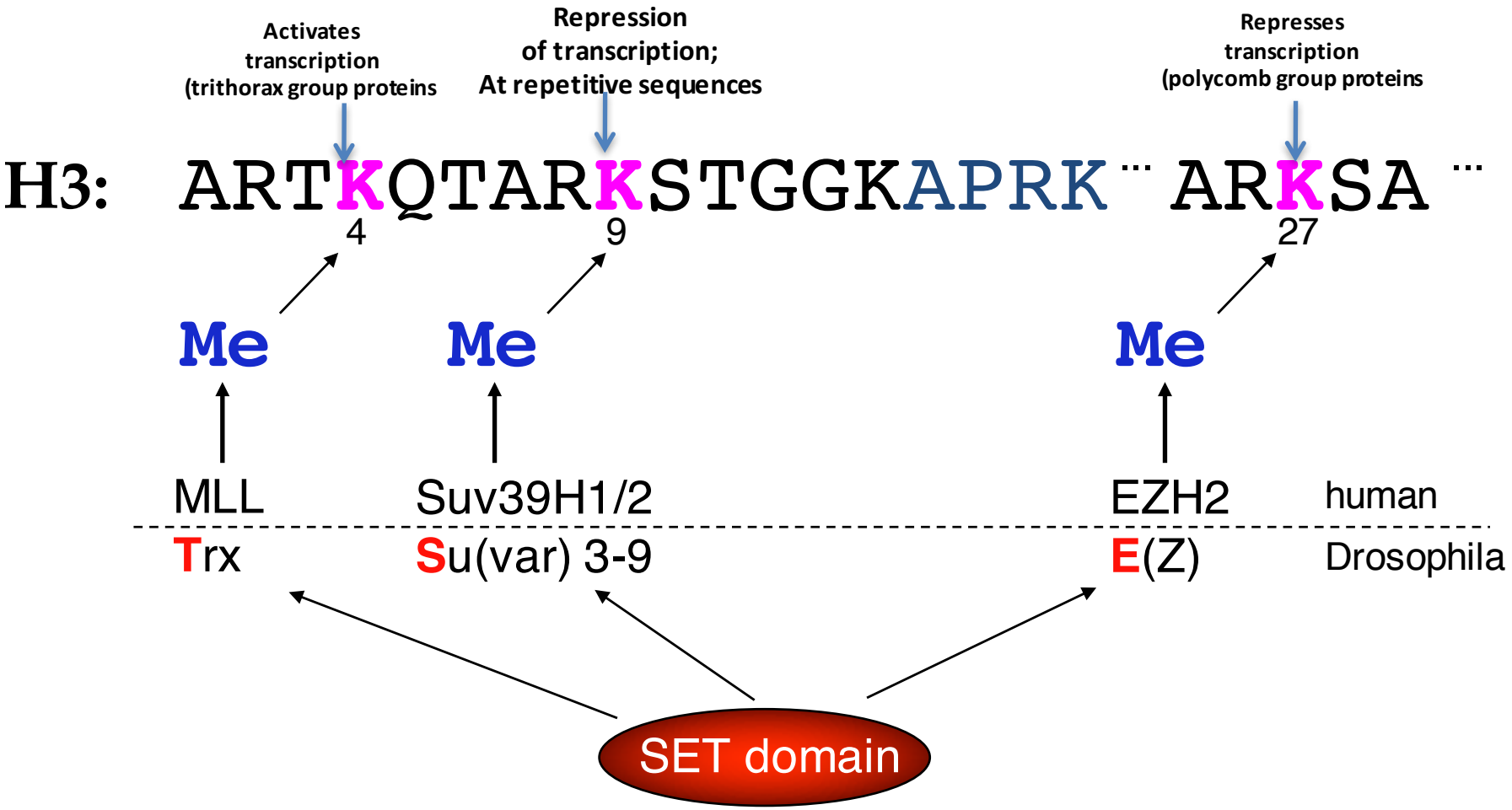
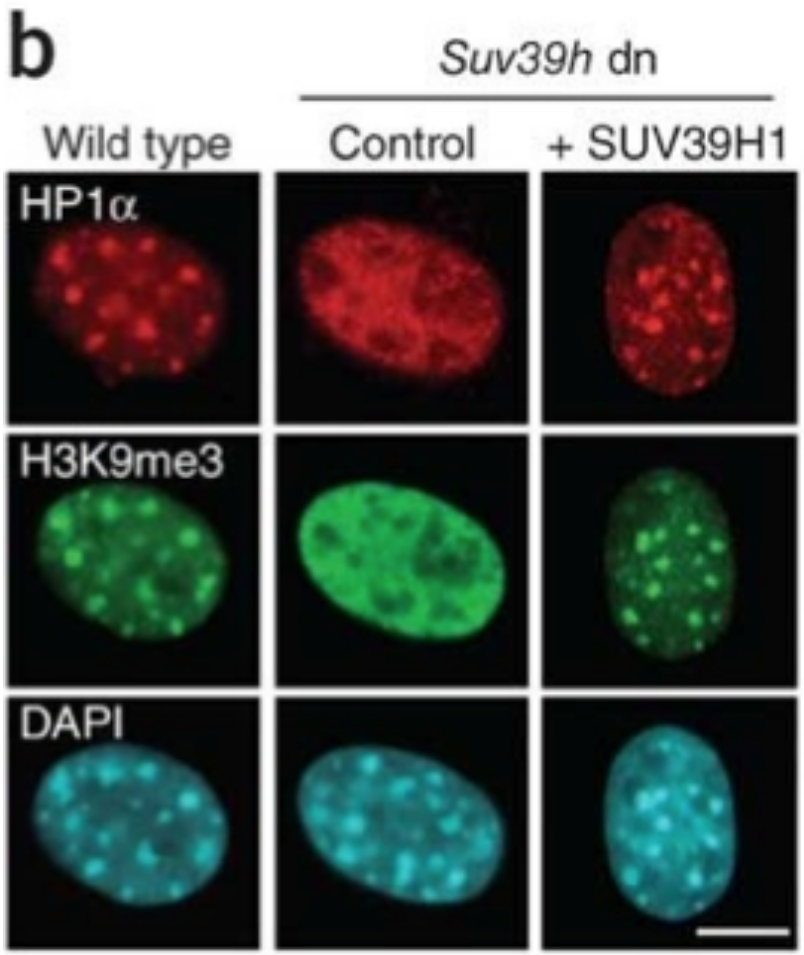


Enzymatic domain is conserved

SET domain protein have sequence specifcity for pepide sequence around target K

- Mutations of some histone methyltransferases → cancer

Post-translational histone modifications can recruit specialized proteins



Problem:

1. How can we detect epigenetic modifications?

-Generation of antibodies that specifically Recognize modified histones (western blotting/Immunofluorescence)
- Mass spectroscopy

Problem:

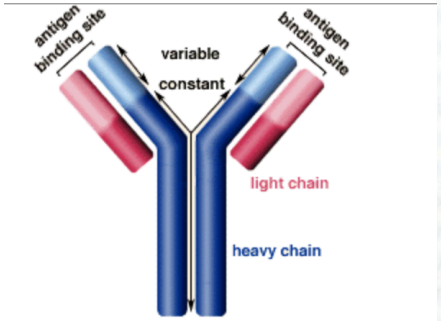
2. How can we locate epigenetic information at defined regions or genes or promoters, etc?

-DAPI intense regions; DNA-FISH probes

-Chromatin immunoprecipitation

--> Detect histone modifications on specific genomic site of interest (i.e promoter of p53)
--> Detect histone modifications at multiple sites or at the entire genome level

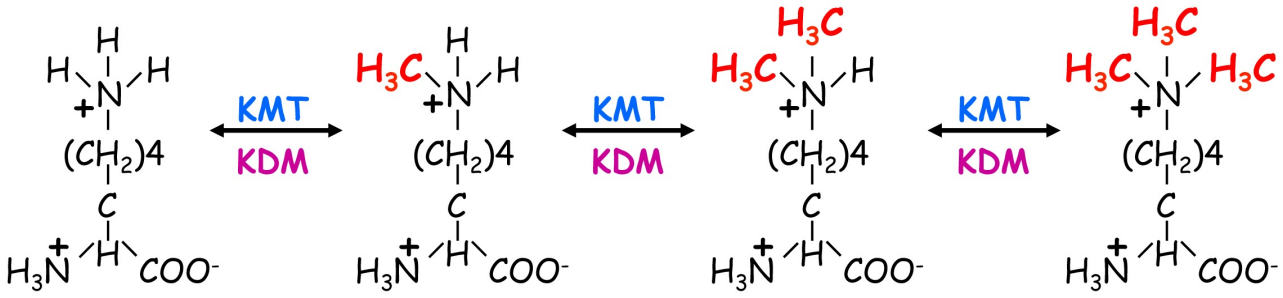
1. Generation of antibodies that specifically recognize modified histones



CAN WE GENERATE MODIIFCATION SPECIFIC ANTIBODIES??

Lysines can be mono-, di- and tri-methyalted
An example: H3K9methylation by Suv39h1

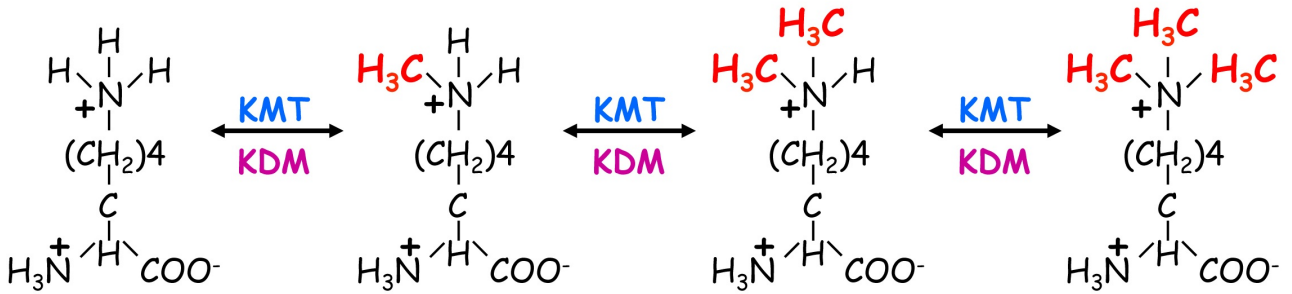
H3K9-specific Lysine-HMTases (KMTases) can mediate mono-, di-, tri-methylation



1. Generation of antibodies that specifically recognize modified histones

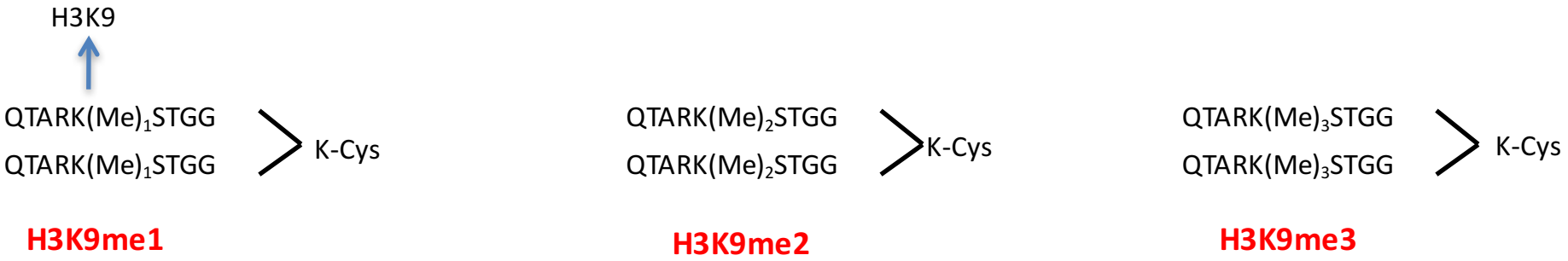
Lysines can be mono-, di- and tri-methylated

H3K9-specific Lysine-HMTases (KMTases) can mediate mono-, di-, tri-methylation



SYNTHESIS OF BRANCHED PEPTIDES FOR ANTIBODY GENERATION

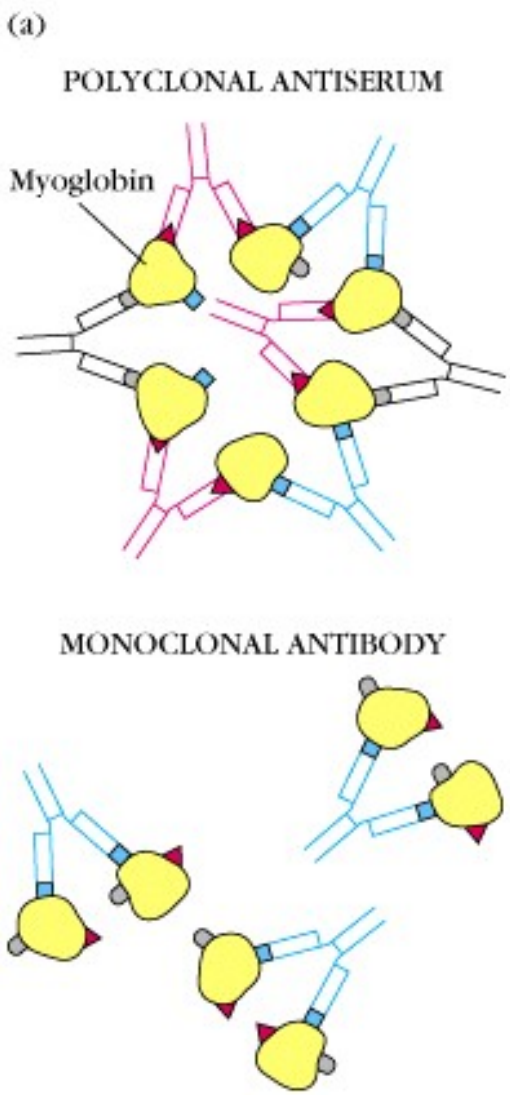
H3: ARTKQTARK$_9$STGGKAPRK



→Branching allows to place histone modifications in close vicinity

→→ resembles high concentration of chromatin modification in the nucleus

Generation of polyclonal/monoclonal antibodies



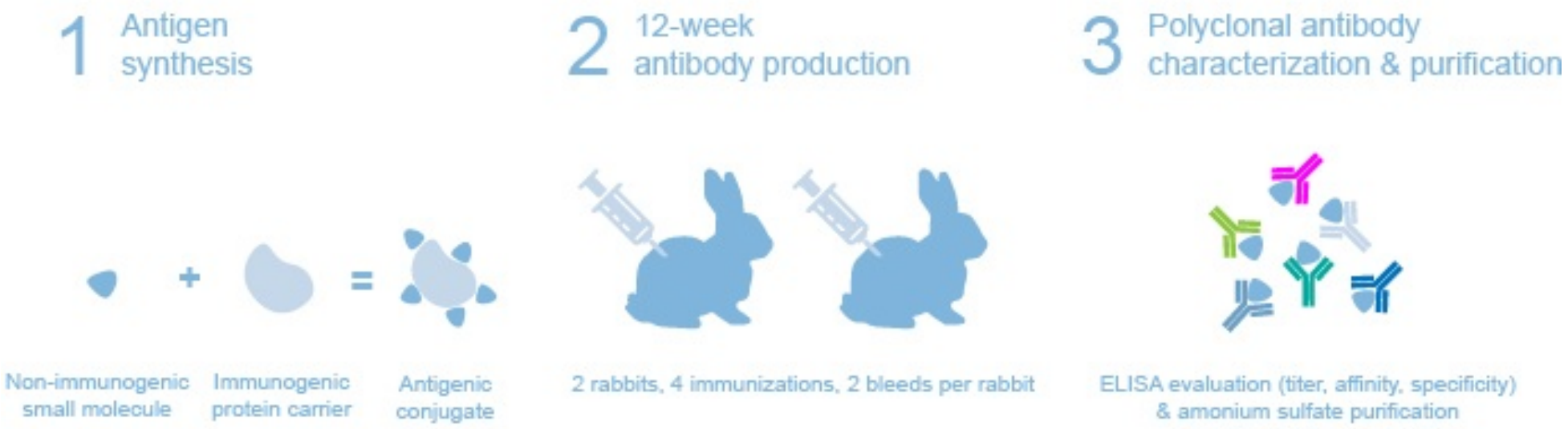
Polyclonal antibodies can form lattices with homogeneous, monomeric protein antigens because each antibody can interact with a different epitope on the antigen.

Monoclonal antibodies do not form lattices with homogeneous, monomeric proteins, because only they can bind to only one epitope on the antigen.

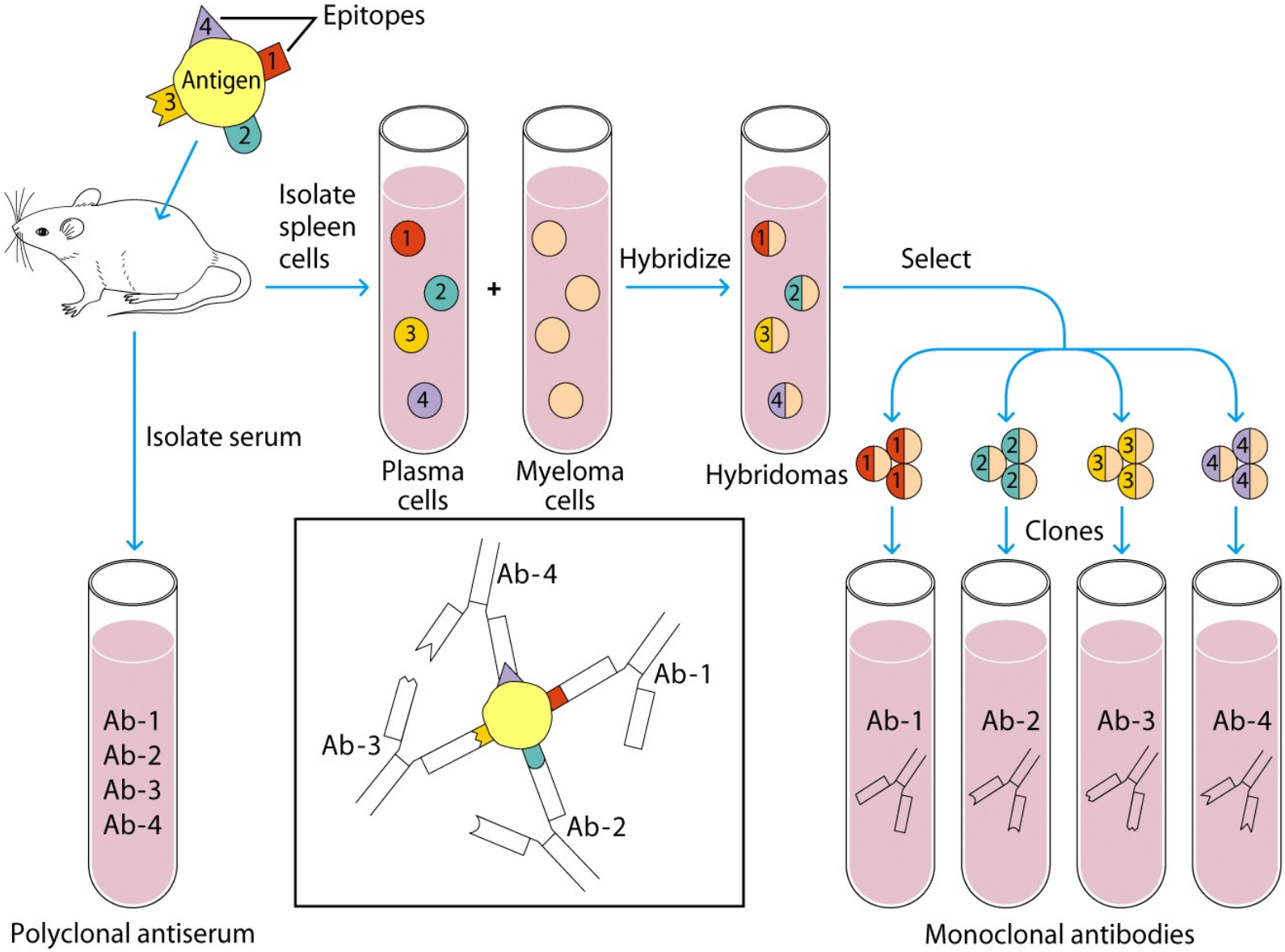
Generation of polyclonal antibodies

QTARK(Me)$_3$STGG
QTARK(Me)$_3$STGG >K-cys

H3K9me3



Generation of monoclonal antibodies



Generation of monoclonal antibodies

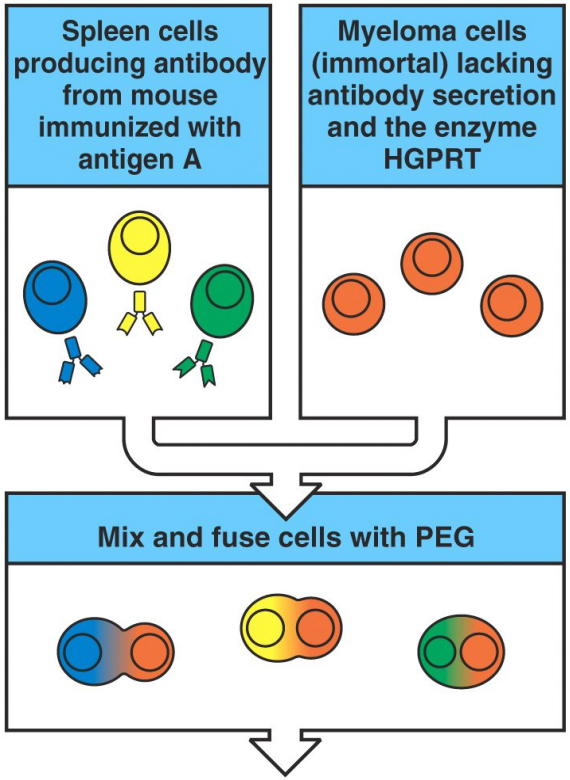


Figure A-14 part 1 of 2 Immunobiology, 6/e. (© Garland Science 2005)

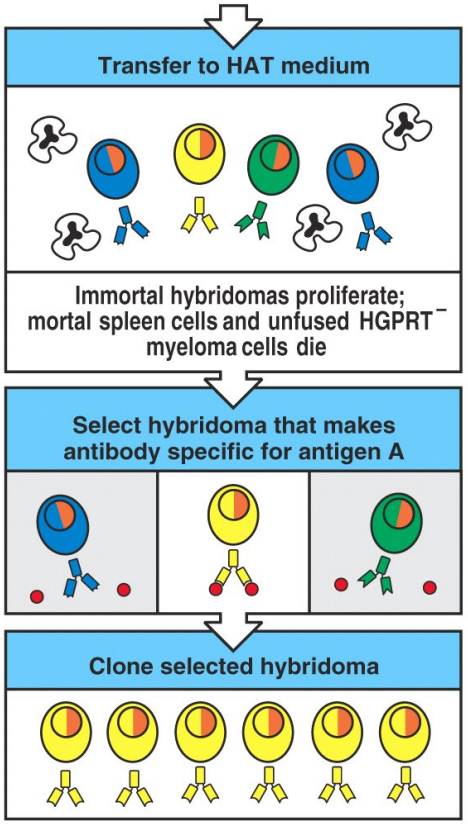
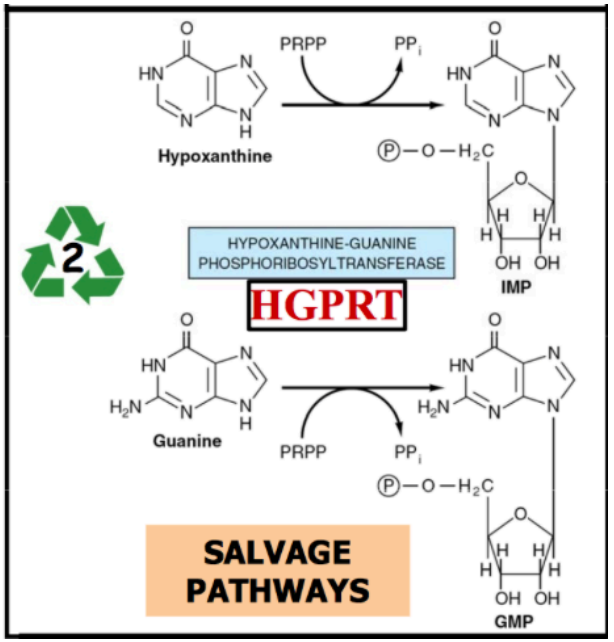


Figure A-14 part 2 of 2 Immunobiology, 6/e. (© Garland Science 2005)



Most purines are recycled rather than degraded. HGPRT can recycle hypoxanthine and guanine to be used in DNA replication

HAT Medium (hypoxanthine-aminopterin-thymidine medium) is a selection medium for mammalian cell culture, which relies on the combination of aminopterin, a drug that acts as a powerful folate metabolism inhibitor by inhibiting dihydrofolate reductase, with hypoxanthine (a purine derivative) and thymidine (a deoxynucleoside) which are intermediates in DNA synthesis. Its binding affinity for dihydrofolate reductase effectively blocks tetrahydrofolate synthesis. This results in the **depletion of nucleotide precursors** and inhibition of DNA, RNA, and protein synthesis. **The trick is that aminopterin blocks DNA de novo synthesis, which is absolutely required for cell division to proceed, but hypoxanthine and thymidine provide cells with the raw material to evade the blockage (the "salvage pathway"), provided that they have the HGPRT gene → rescue of DNA replication**

HAT medium is often used for preparation of monoclonal antibodies. This process is called Hybridoma technology. Laboratory animals (e.g., mice) are first exposed to an antigen against which we are interested in isolating an antibody. Once splenocytes are isolated from the mammal, the B cells are fused with HGPRT negative, immortalized myeloma cells using polyethylene glycol or the Sendai virus. Fused cells are incubated in the HAT medium. Aminopterin in the medium blocks the de novo pathway. Hence, unfused myeloma cells die, as they cannot produce nucleotides by de novo or salvage pathway. Unfused B cells die as they have a short lifespan. In this way, only the B cell-myeloma hybrids survive. These cells produce antibodies (a property of B cells) and are immortal (a property of myeloma cells). The incubated medium is then diluted into multiwell plates to such an extent that each well contains only 1 cell. Then the supernatant in each well can be checked for desired antibody. Since the antibodies in a well are produced by the same B cell, they will be directed towards the same epitope, and are known as monoclonal antibodies.

Generation of monoclonal antibodies

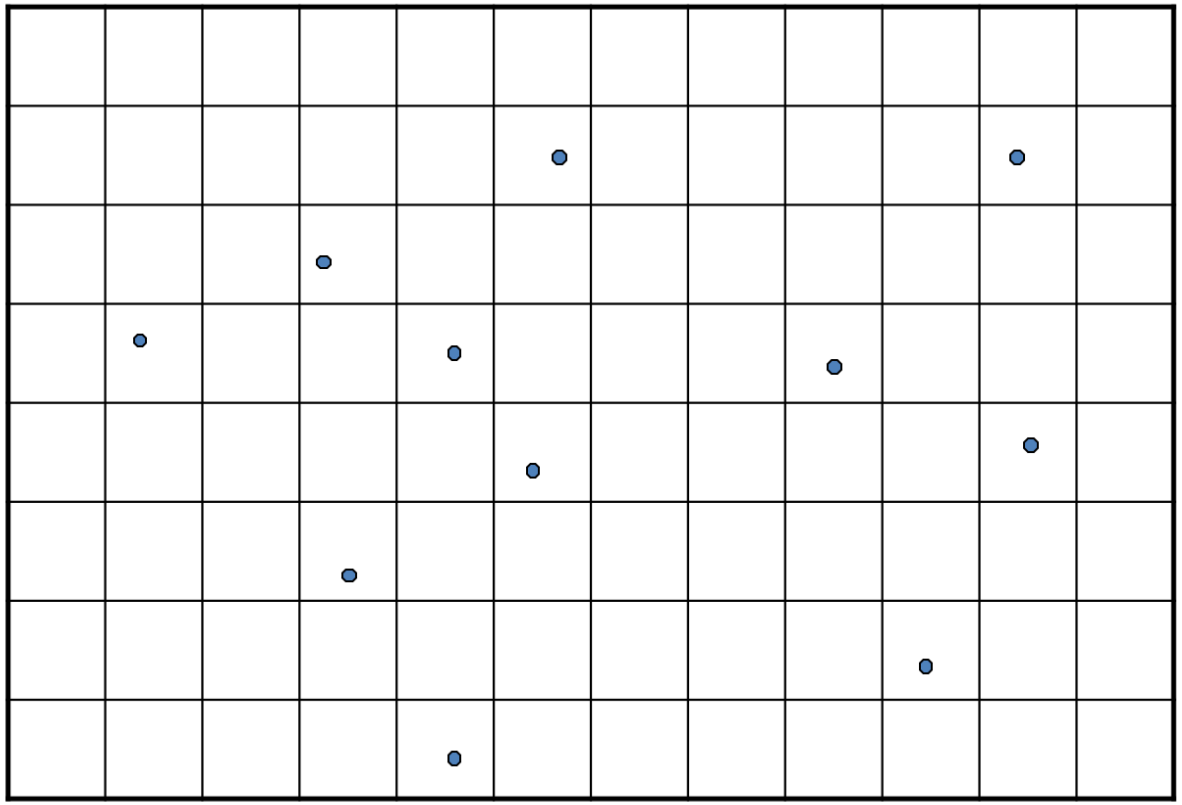
Cloning hybridomas from fusion event

1. Plate at limiting dilution (<1 cell/well) in 96 well plates.

- for example: volume per well: 100 microliter
- use fused cells and prepare cell suspession of ca. 1 cell/ml (0,1 cell per100ul well → 1 cell every 10 wells)

2. Allow clones to expand in 96 well = cell clone or cell line (a population of hybridoma cells derived from a single cell = genetically identical) → Hybridoma line

3. Further expand positive well and test for production of antibody of desired specificity in culture supernatant



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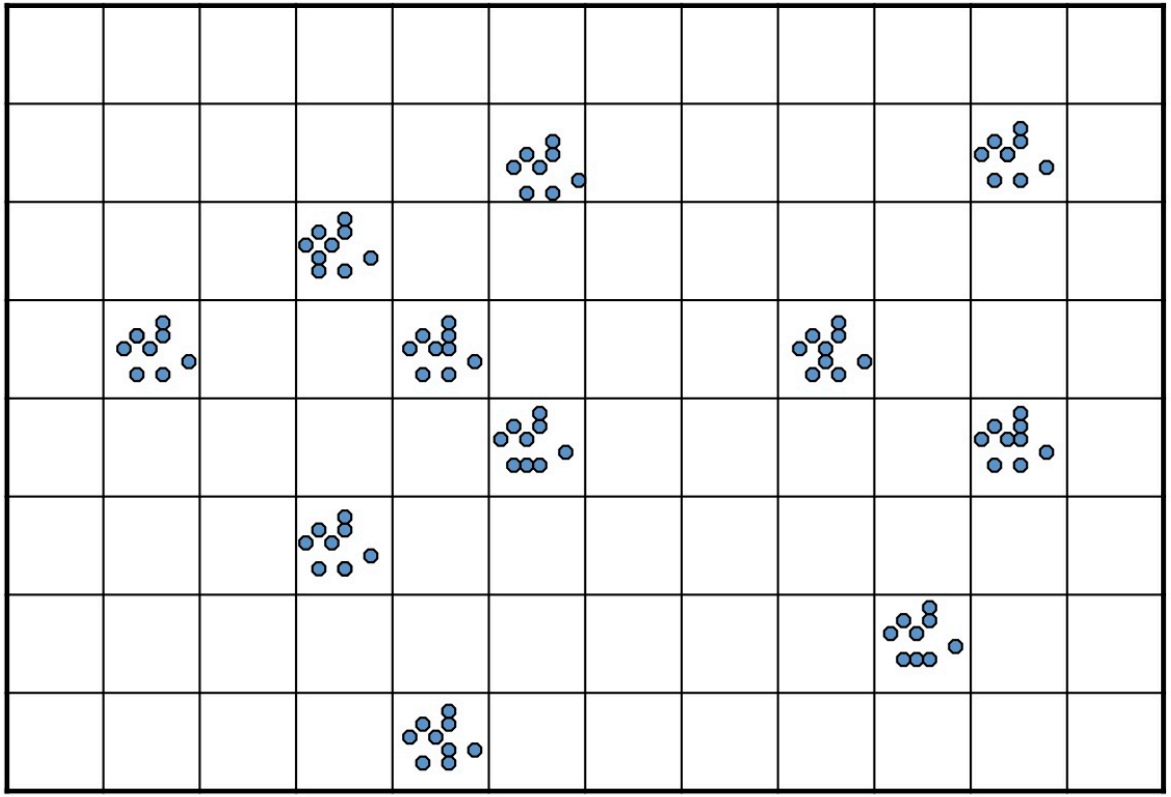
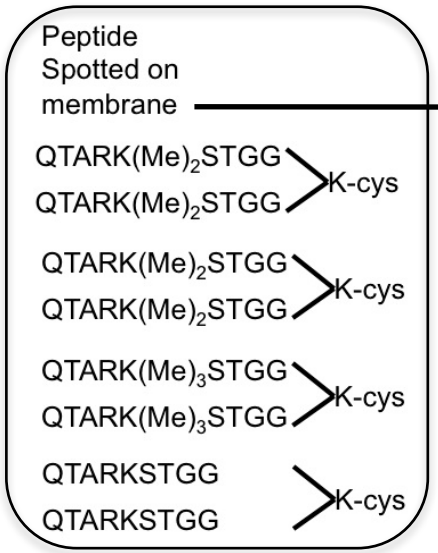
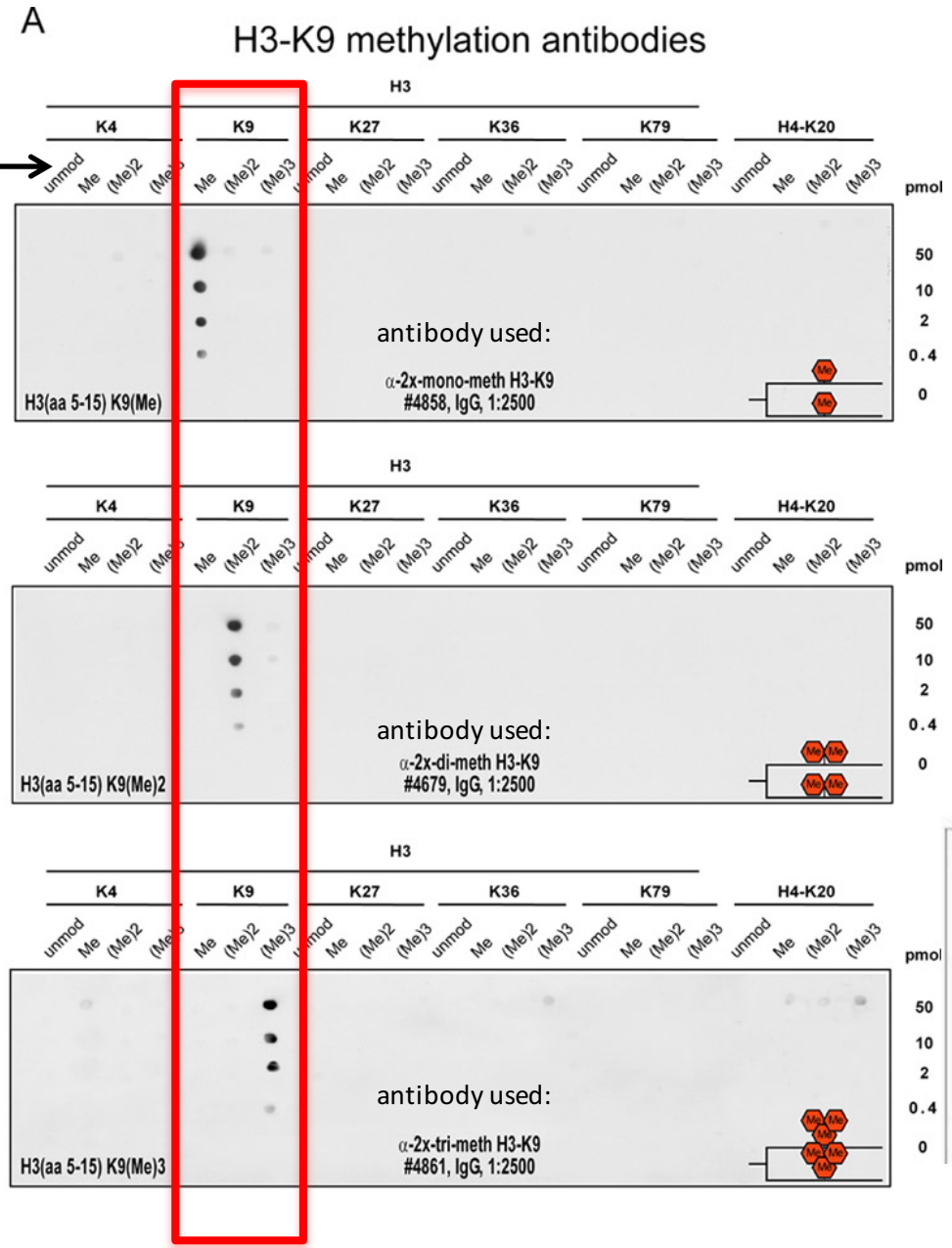


-Hybridoma cells secrete antibody into cell culture medium
-Purification of antibody
-Validation of antibody

Validation of antibodies





Validation by dot-blot
(pure antigen spotted on membrane)

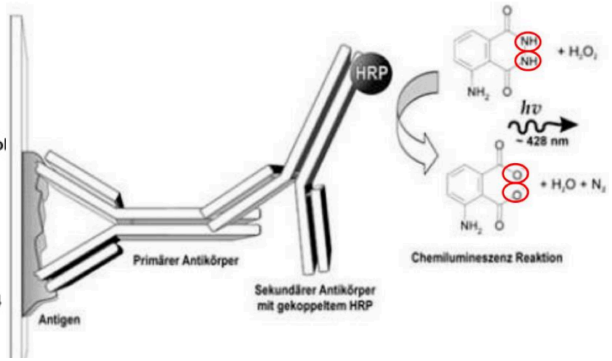
1. Spot branched peptides at different concentration on membrane

2. Incubate with respective Antibody

3. Incubate with secondary Antibody that is coupled with horseradish peroxidase

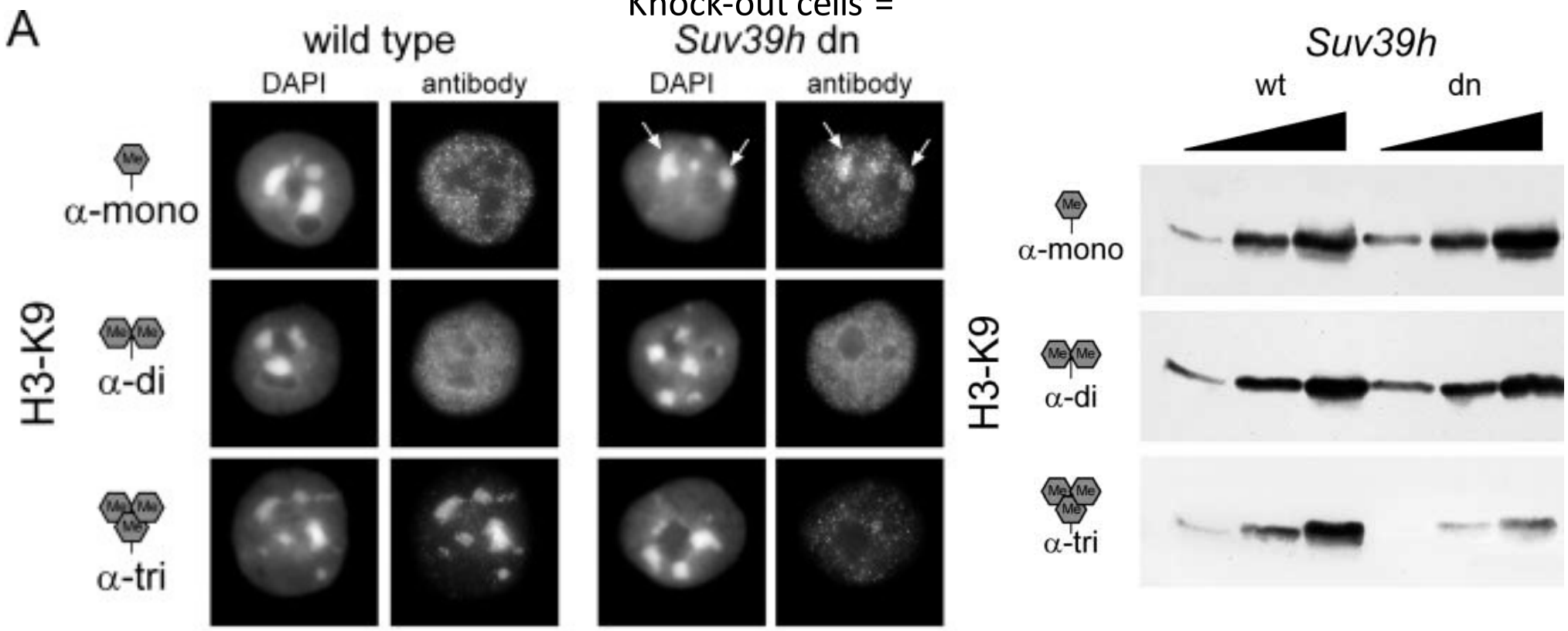
4. Add substrate for Horseradish peroxidase

5. Develop



Obtaining evidence for the specificity of histone modifying enzymes → in cells

Suv39h1/Suv39h2
Knock-out cells =

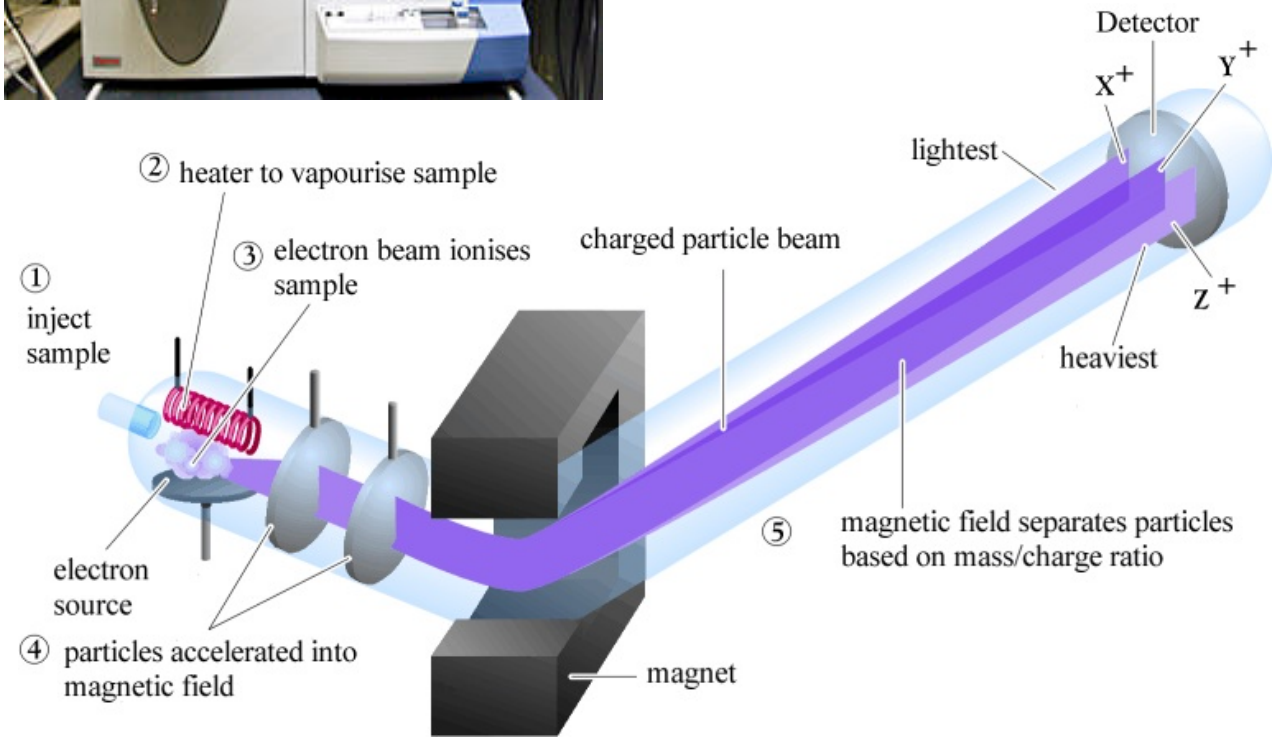


or Suv39h1 siRNA

In Suv39h dn cells, H3K9me2 and H3K9me2 are still present.
Suv39h1 is a H3K9 specific HMTase that is required to establish the TRI-METHYLATION of H3K9 in mammalian cells

Note, that in the absence of H3K9me3, H3K9me1 is localized at DAPI rich regions

2. Studying histone modifications by mass spectrometry



In a typical MS procedure proteins are ionized, for example by bombarding it with electrons. This may cause some of the sample's molecules to break into charged fragments.

These ions are then separated according to their mass-to-charge ratio, typically by accelerating them and subjecting them to an electric or magnetic field:

Ions of the same mass-to-charge ratio will undergo the same amount of deflection. Ions with different mass-to-change ratio will show different deflection

→mono-methylated H3K9 has different defection than di-or tri-methylated H3K9

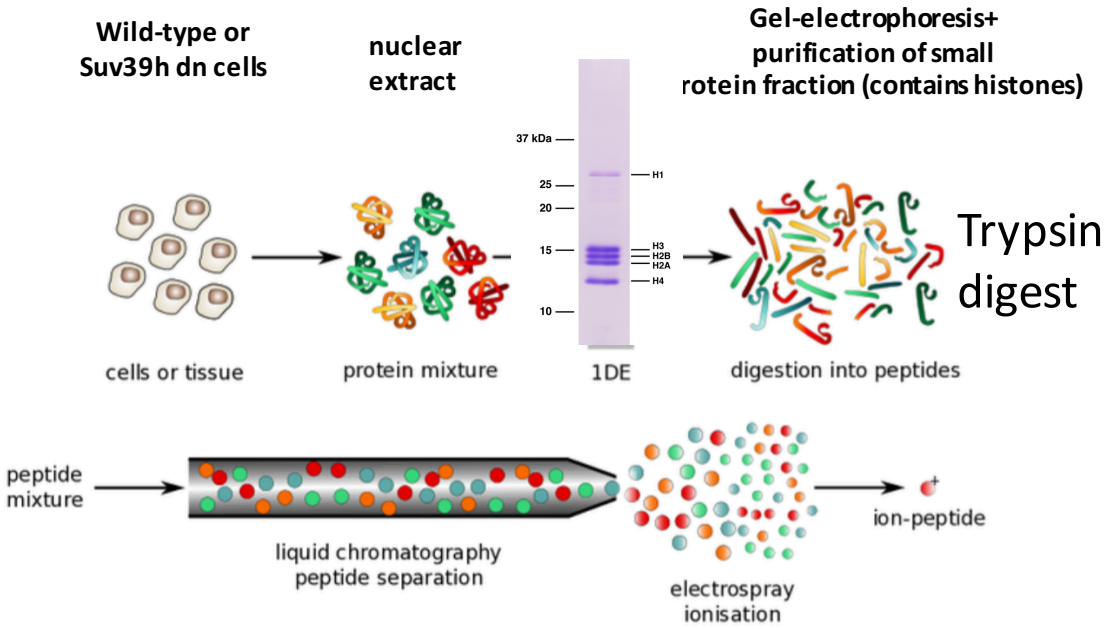
The ions are detected by a mechanism capable of detecting charged particles, such as an electron multiplier. Results are displayed as spectra of the relative abundance of detected ions as a function of the mass-to-charge ratio.

The molecules in the sample can be identified by correlating known masses to the identified masses or through a characteristic fragmentation pattern.

MW of all amino acids and all their possible modifications are know = identifiable by mass-to-change ration: also when present in a series of aminoacids

MOST IMPORTANT: provide a sample with defined peptide fragments → achieved by digest with proteases that cut peptides at defined positions (comparable with restriction enzymes)

2. Studying histone modifications by mass spectrometry



Digestion of fraction containing histones with proteases creates a "library" of small peptides that are derived from histones:
Protease= Trypsin
Trypsin cleaves after every Lysine (K) and Arginine (R)

IMPORTANT: we know already All proteins and the amminoacid sequence of all proteins of a cell!!!
That means we can predict all possible small peptide sequences that result from a trypsin cleavage

Highly informative fragments

Downstream MS analysis can focus on these sites (same holds true for ohter histones!!)

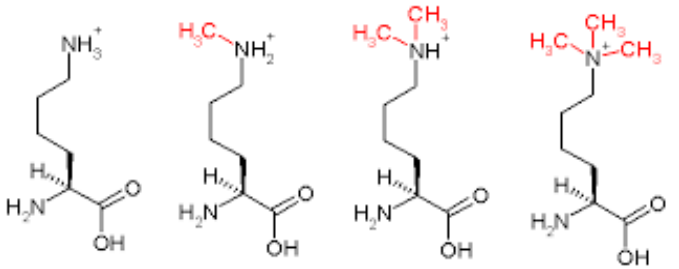
Cutting after K and R

Arows indicate trypsin target sites for cleavage

Histone modifications change m/z ratio in mass spec experiments

Table of amino acid residues .

Symbol	Structure	Mass (Da
Ala A	-NH.CH.(CH_3).CO-	71.0
Arg R	-NH.CH.[$(CH_2)_3$.NH.C(NH).NH_2].CO-	156.1
Asn N	-NH.CH.(CH_2CONH_2).CO-	114.0
Asp D	-NH.CH.(CH_2COOH).CO-	115.0
Cys C	-NH.CH.(CH_2SH).CO-	103.0
Gln Q	-NH.CH.($CH_2CH_2CONH_2$).CO-	**128.1**
Glu E	-NH.CH.(CH_2CH_2COOH).CO-	129.0
Gly G	-NH.CH_2.CO-	57.0
His H	-NH.CH.($CH_2C_3H_3N_2$).CO-	137.1
Ile I	-NH.CH.[CH.(CH_3)CH_2.CH_3].CO-	**113.1**
Leu	-NH.CH.[$CH_2CH(CH_3)_2$].CO-	**113.1**
Lys K	-NH.CH.[$(CH_2)_4NH_2$].CO-	**128.1**
Met M	-NH.CH.[$(CH_2)_2$.SCH_3].CO-	131.0
Phe F	-NH.CH.(CH_2Ph).CO-	147.1
Pro P	-NH.$(CH_2)_3$.CH.CO-	97.1
Ser S	-NH.CH.(CH_2OH).CO-	87.0
Thr T	-NH.CH.[CH(OH)CH_3].CO-	101.0
Trp W	-NH.CH.[$CH_2.C_8H_6N$].CO-	186.1
Tyr Y	-NH.CH.[$(CH_2).C_6H_4$.OH].CO-	163.1
Val V	-NH.CH.[$CH(CH_3)_2$].CO-	99.1



KSTGGK: MW= 577 g/mol CH_3 : MW= 15 g/mol

Kme_3STGGK: MW=577 – 3 + 45 = 619 g/mol

Kme_2STGGK: MW= 577 – 2 + 30 = 605 g/mol

Kme_1STGGK: MW= 577 – 1 + 15 = 591g/mol

Protonation changes MW in a defined manner

Molecular weight

ionization

For details: An Introduction to Mass Spectrometry
http://www.astbury.leeds.ac.uk/facil/MStut/mstutorial.htm

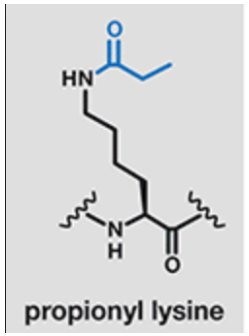
2. Studying histone modifications by mass spectrosmetry

PROBLEM:

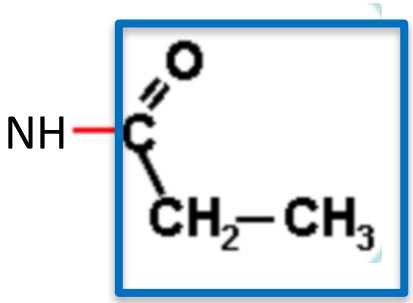
1.Trypsin cleavage after K is not complete because me2 or me3 on some lysines block block cutting by Trypsin

2. K is frequent in histone tails --> result many cleavages (mostly at me0, me1) → many small heterogeneous peptides → difficult to analyze

Trypsin can cleave after Arg (R)
Trypsin can cleave after K-unmethylated; K-me1
Trypsin cannot cleave after K-me2; K-me3

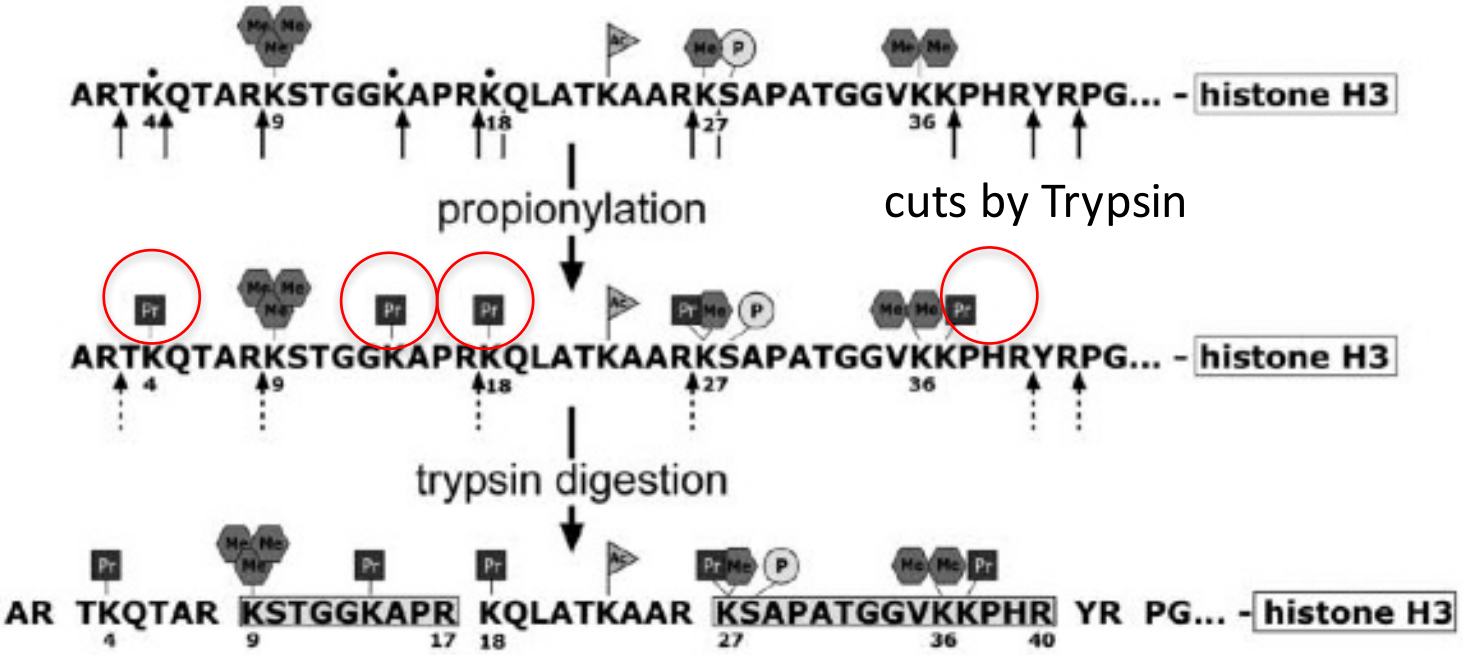


Proprionyl group



SOLUTION: In vitro Propionylation of unmodified or mono-methylated lysine prevents trypsin cleavage
→ now Trypsin can only cut after Arginine.
→ this allows a uniform cleavage of histone tails
→ creates a mass:charge ratio that allows to differentiate between fragments carrying me0, me1, me2 or me3 marks (me0 + 1x propionyl group; me1 + 1x propionyl group; me2 +0 propionyl group; me3 + 0x propionyl groups)

2. Studying histone modifications by mass spectrometry



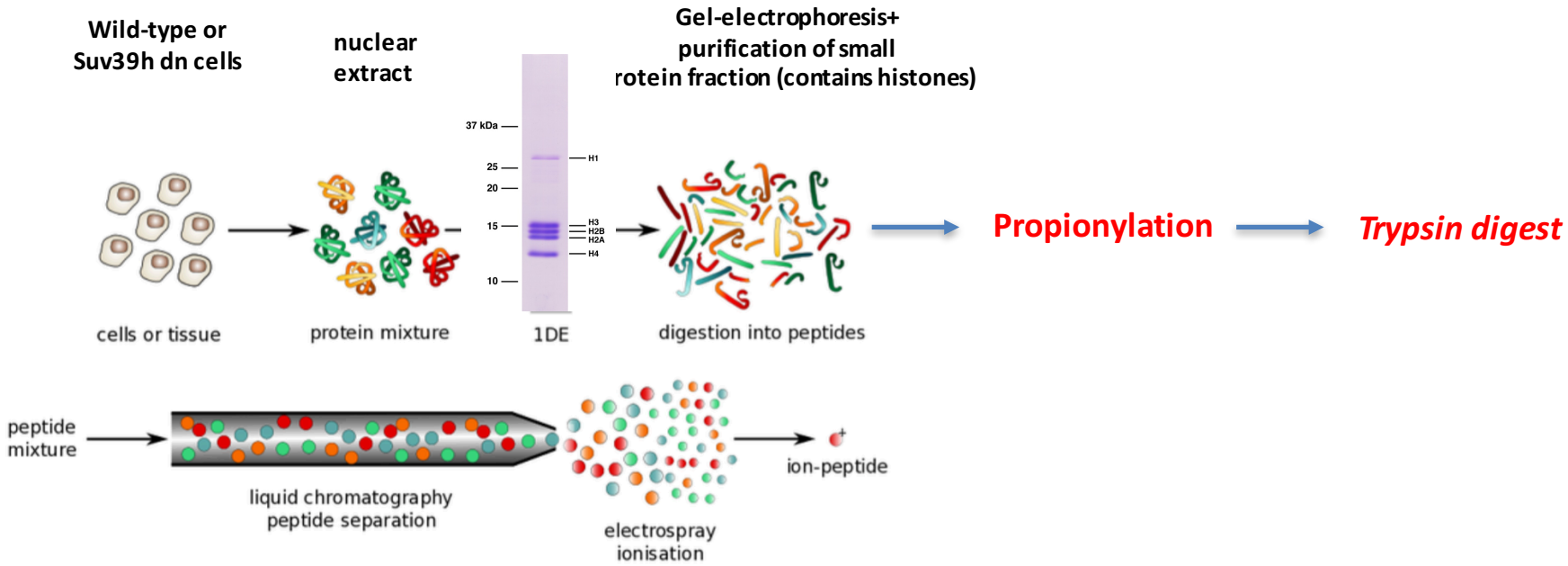
propionylation

bigger and homogeneous fragments

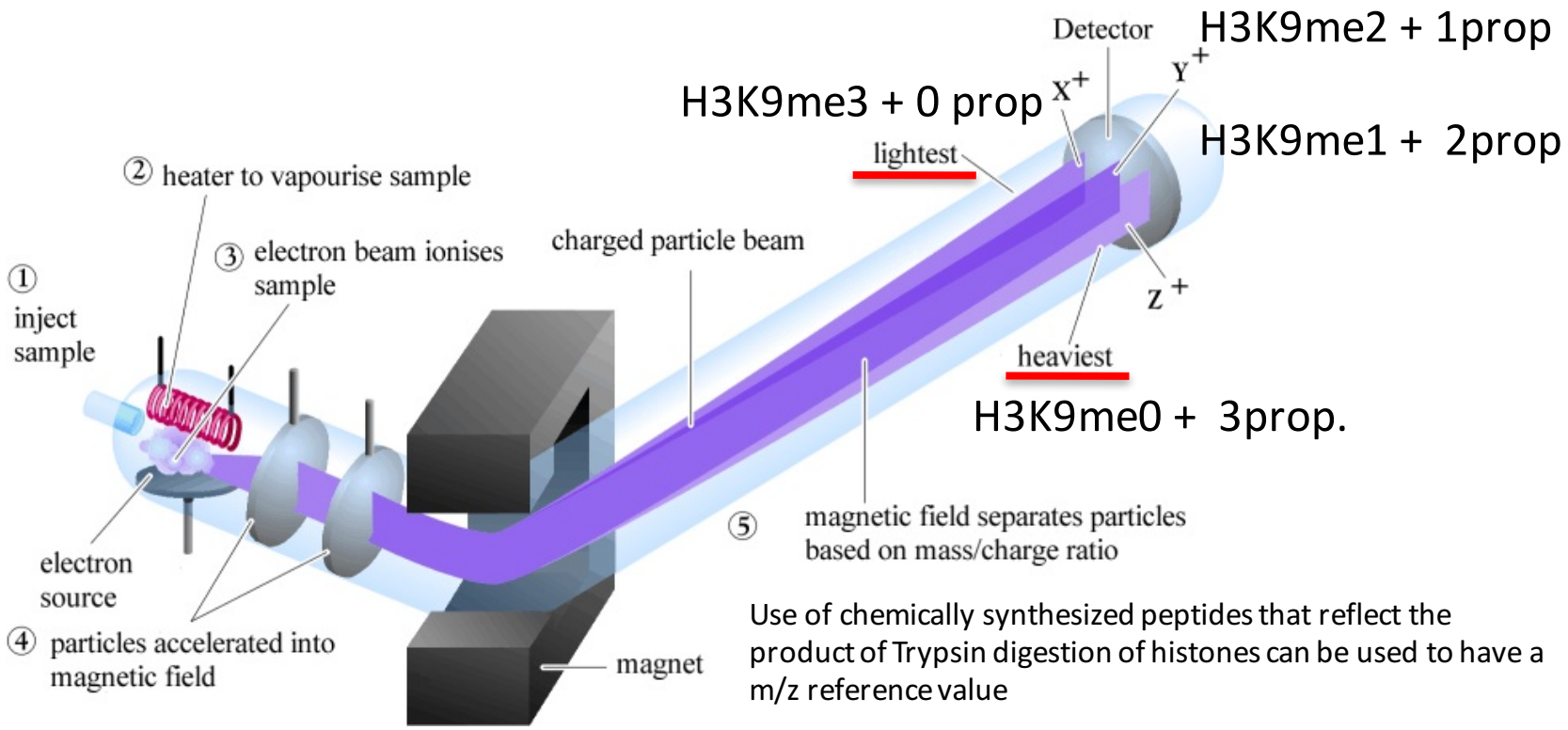
TRYPSIN cuts ONLY at Arg by Trypsin after propionylation

Now Trypsin can only cut after Arginine. This allows a uniform cleavage of histone tails
The number of methyl-groups/propinyl groups at the H3 peptide K9 – R17 decides on the deflection of this specific peptide in mass spectrometry.
Important: All peptides cut at Arg by Trypsin are analyzed contemporarily!!!!

2. Studying histone modifications by mass spectrometry



2. Studying histone modifications by mass spectrometry

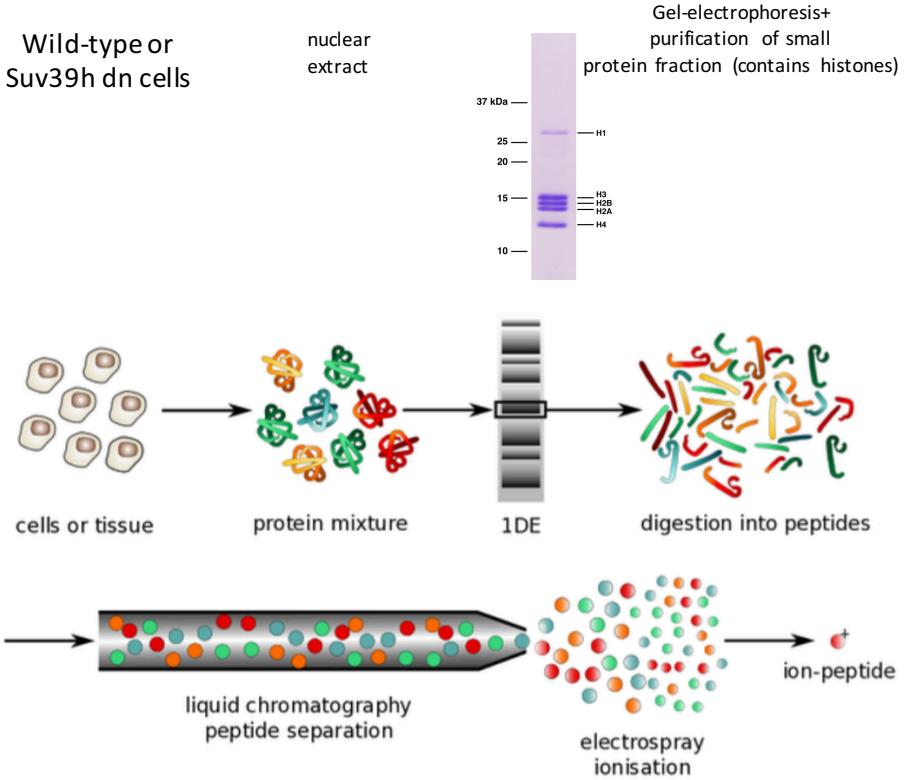


Use of chemically synthesized peptides that reflect the product of Trypsin digestion of histones can be used to have a m/z reference value

The method allows to quantify the numbers of H3K9me0, H3K9me1 H3K9me2 and H3K9me3 in a sample → We can calculate the % of each histone modification in the given sample.

Important: The analysis is not limited to H3K9 → other histone modifications can be quantified in the same analysis

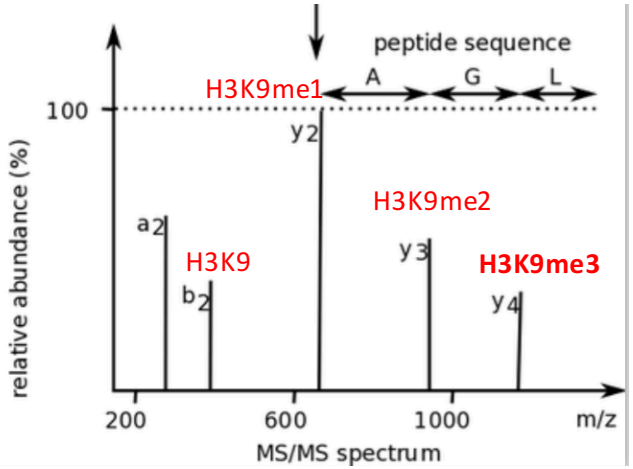
2. Studying histone modifications by mass spectroscopy



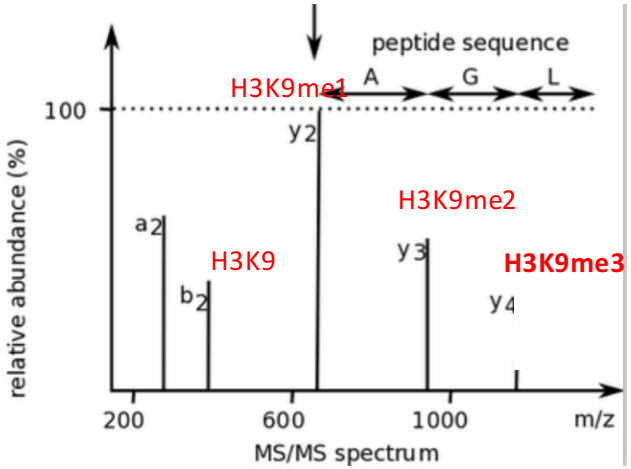
Digestion of fraction containing histones with proteases creates a "library" of small peptides that are derived from histones:
Protease= Trypsin
Trypsin cleaves after every Lysine (K) and Arginine (R)

IMPORTANT: we know already All proteins and the amminoacid sequence of all proteins of a cell!!!
That means we can predict all possible small peptide sequences that result from a trypsin cleavage

MS →

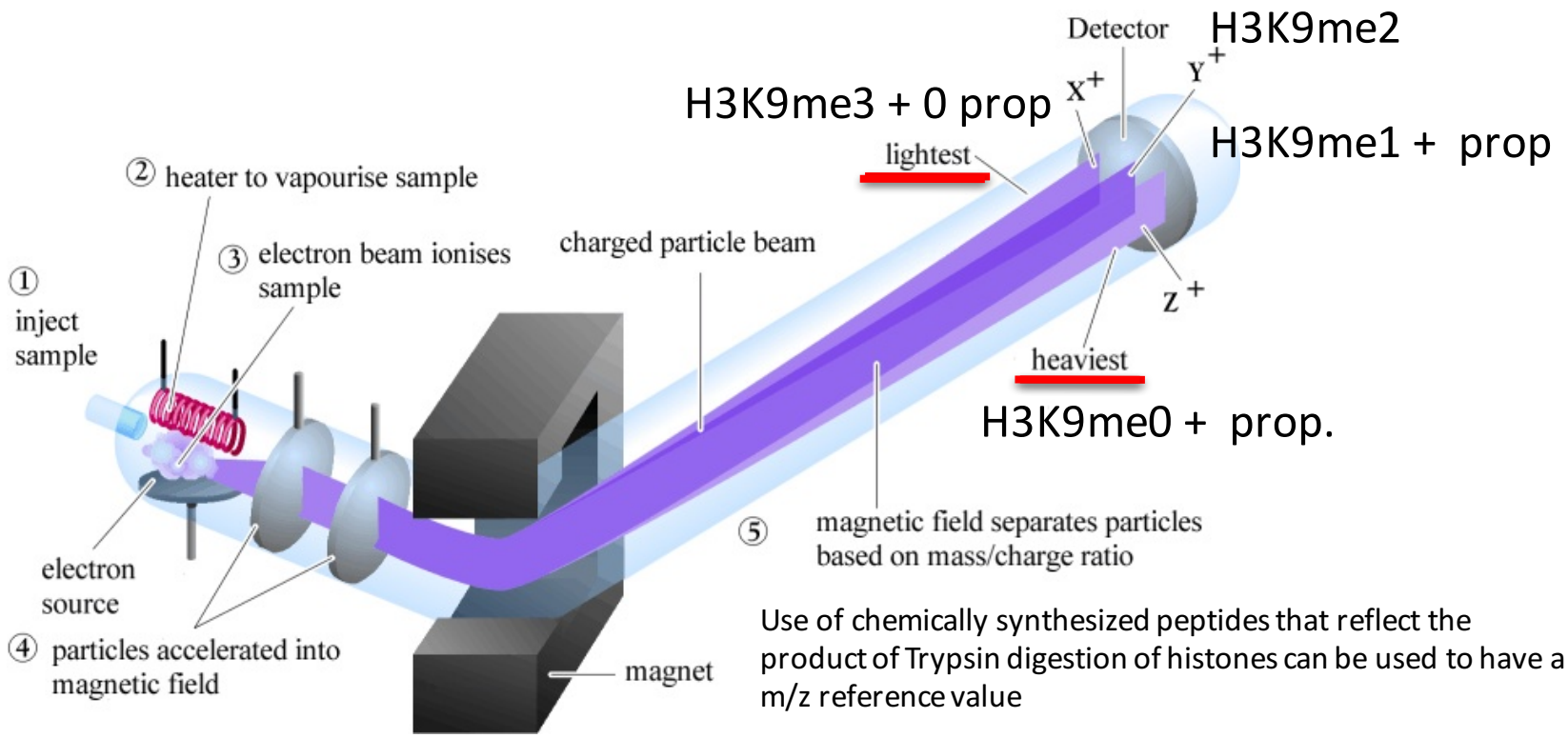


WT profile: Mass/charge ratio



Suv39DN profile: Mass/charge ratio

2. Studying histone modifications by mass spectroscopy

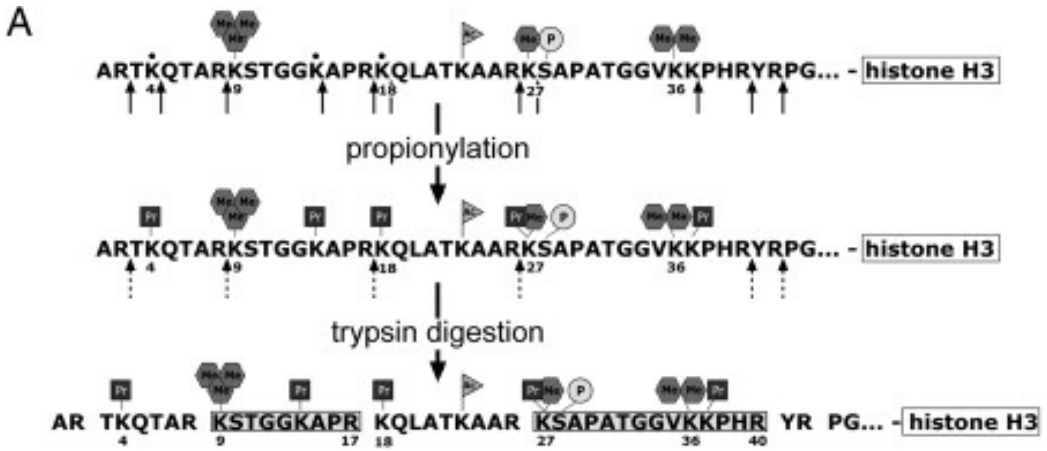


Use of chemically synthesized peptides that reflect the product of Trypsin digestion of histones can be used to have a m/z reference value

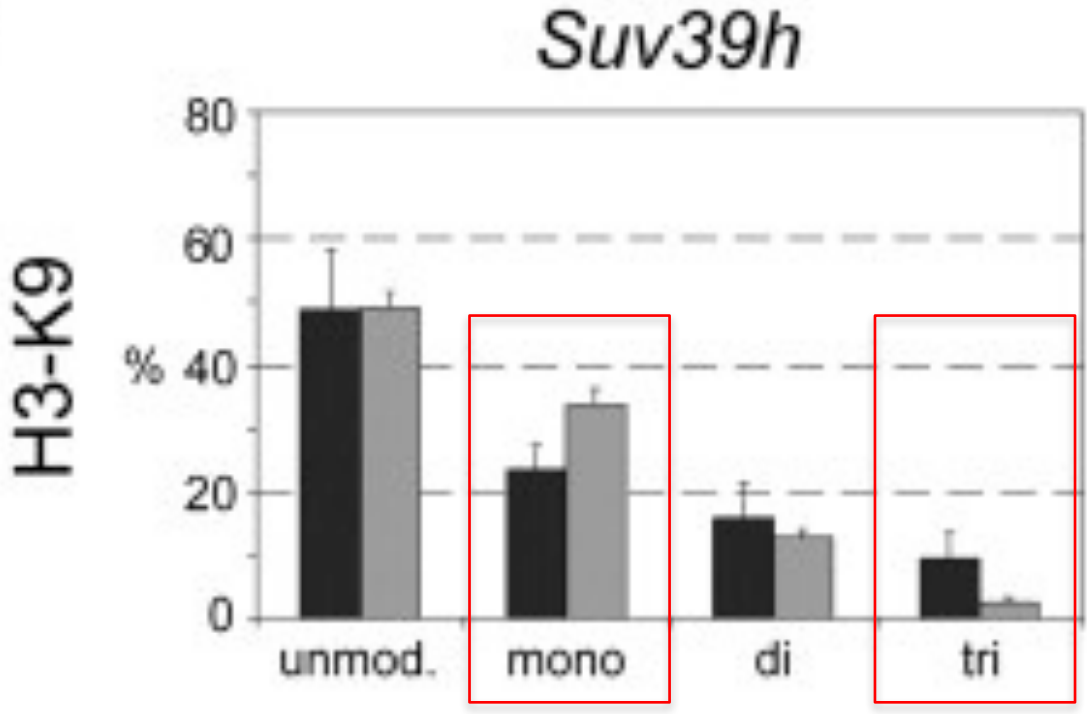
The method allows to quantify the numbers of H3K9me0, H3K9me1 H3K9me2 and H3K9me3 in a sample → We can calculate the % of each histone modification in the given sample.

Important MS is a proteomics approach: The analysis is not limited to H3K9 → all other histone modifications can be quantified in the same analysis. Remember: the histone proteins were isolated prior to MS.
This allows also to discover possible regulatory loops between different histone modifications!!! (for this you always need 2 biologically different samples: genetic model; differentiation, treatment, etc…)

2. Quantifying histone modifications by mass spectroscopy



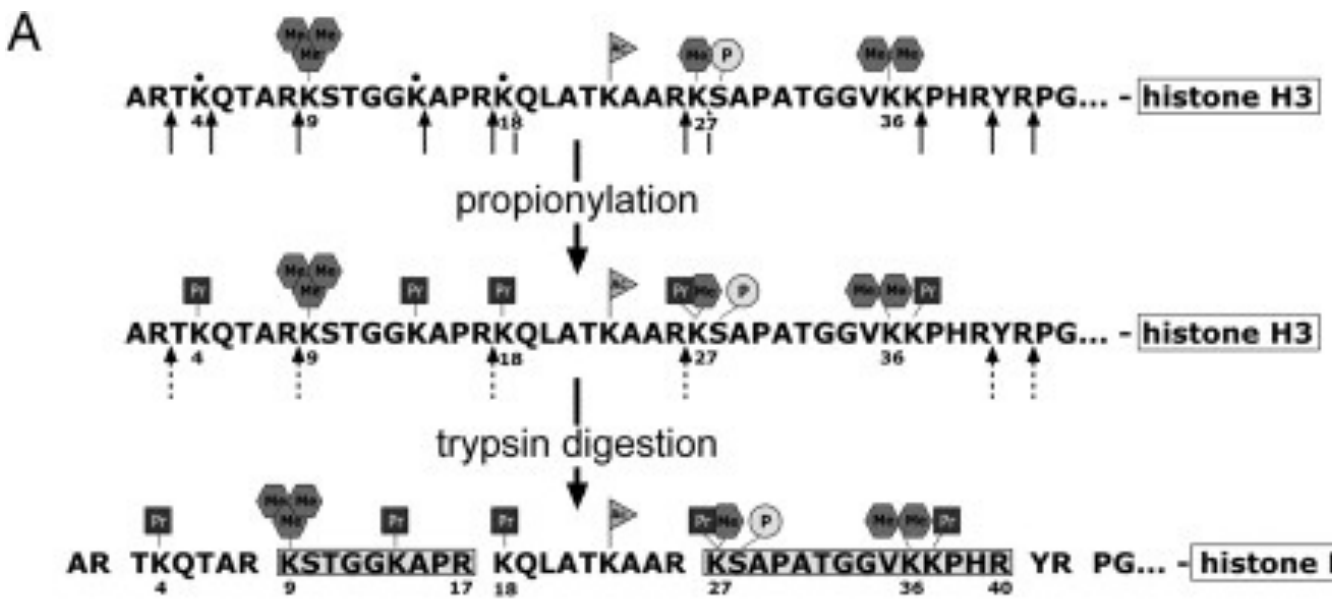
Suv39h1 and Suv39h2 ensure H3K9me3.
H3K9me1 is a preferred substrate for Suv39h1/h2
Suv39h1/h2 methylate H3K9me1 Until reaching the tri-methylated state



Wild-type

Suv39h double null (dn)

2. Quantifying histone modifications by mass spectroscopy



Very short peptides, Non homogenouse digest - difficult to analyse in MS

Propionylation of unmodified Ks

Propionylation of unmodified Ks

1. Identifying the enzymatic activity of a histone modifying enzymes (LOF of epigenetic writer)

2. Identification of “modifiable” amminoacids in histone tails

3. Functional link between histone modifications

4. Defining the ENTIRE epigenetic status of a cell type (for example: differentiated/stem cell)

5. Quantitative information on histone modifications (%)

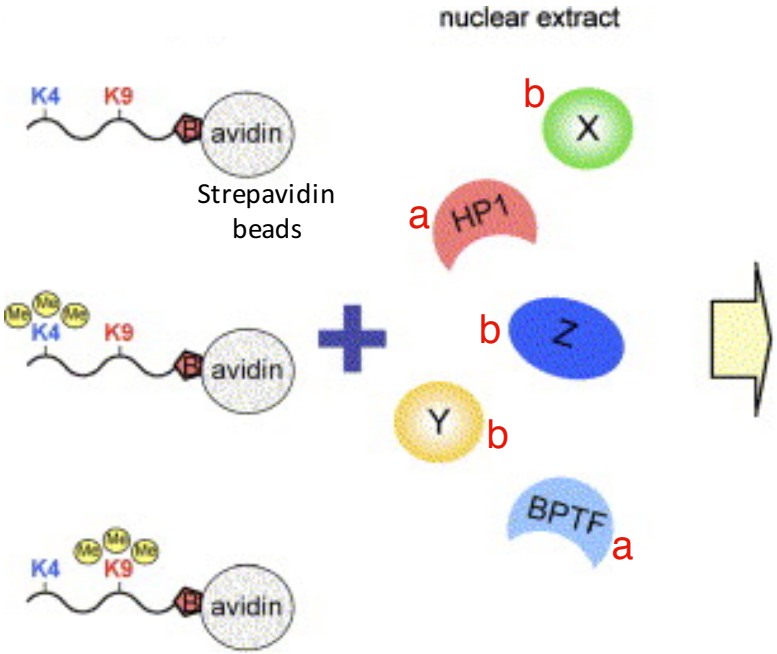
HOW CAN EPIGENTIC READERS BE IDENTIFIED?

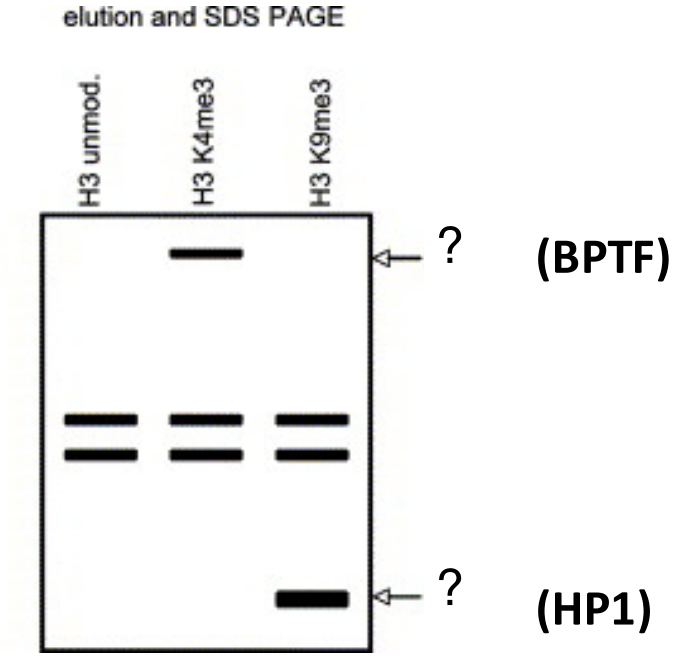
Identifying methyl-H3 binding proteins

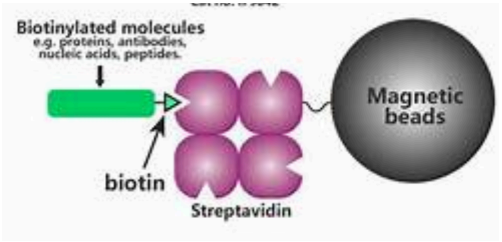
- histone peptide pulldown assay: protein identificiation

Chemically synthesized peptides, fused to biotin

nuclear extract





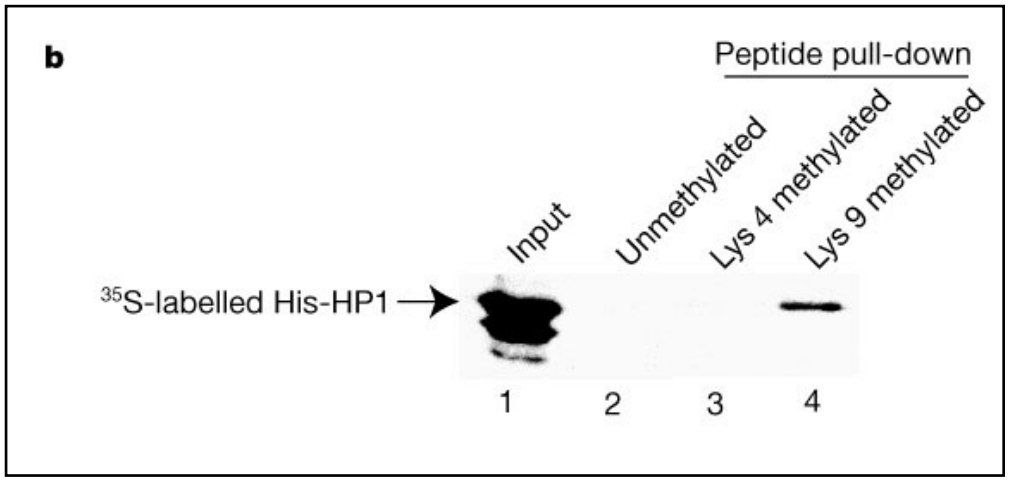


→ stain proteins in gel;
→ cut out specific band,
→ identify protein by Mass Spec

What are the target sites for Suv39h1 and H3K9me???

- histone peptide pulldown assay: validation of interaction and functional analysis

- Using the peptide pull-down assay, it was found that Lys9-methylated H3 binds to heterochromatin protein 1 (HP1)



Pull-down assay:
-Couple biotinylated histone tail-peptides, carrying specific modifications (methylated, or unmethylated) to stretptavidine coated resin

-Incubate with recombinant ^{35}S-labelled HP1, produced in E-coli

-Wash resin

-Elute bound proteins, run gel and make radiography

- HP1 is a protein previously identified to be enriched in and important for heterochromatin assembly

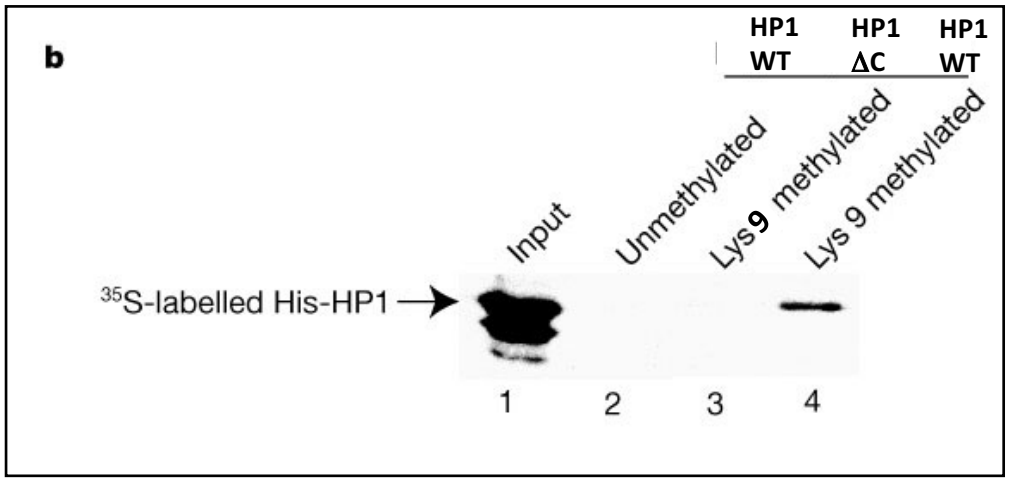
- Lys9-methylated H3 binds to HP1 via the chromodomain motif in HP1

Lys9-methylated H3 binds to the conserved motif called chromodomain

Bannister et al, Nature, 2001

What are the target sites for Suv39h1 and H3K9me???

- histone peptide pulldown assay: validation of interaction and functional analysis



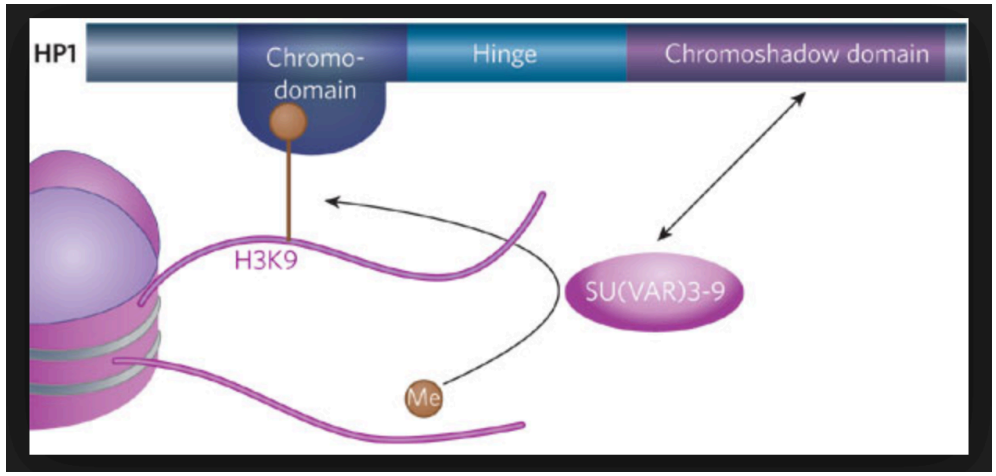
Pull-down assay:

-Couple biotinylated histone tail-peptides, carrying specific modifications (methylated, or unmethylated) to stretptavidine coated resin

-^{35}S-labelled mutant versions of HP1: ΔC; ΔCS; ΔH

-Wash resin

-Elute bound proteins, run gel and make radiography



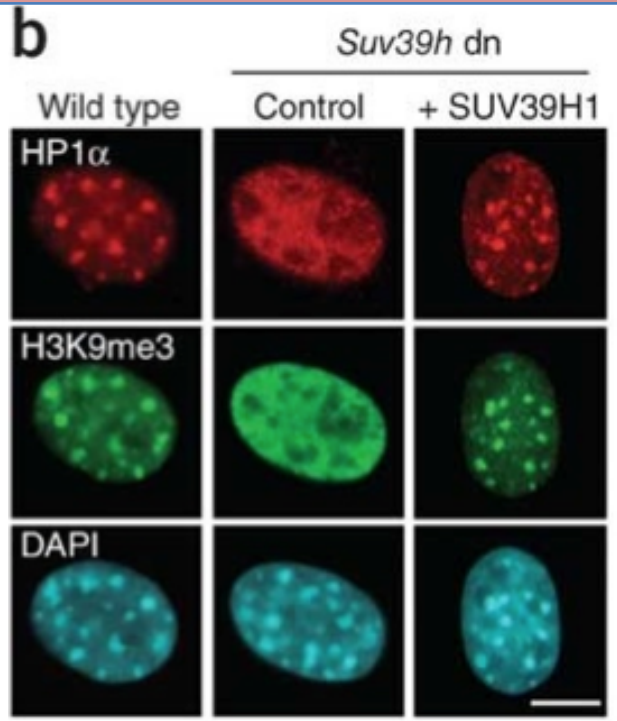
Delta C domain (chromodomain) doesn't bind:
= binds modified histone tail

Lys9-methylated H3 binds to the conserved motif called chromodomain

Bannister et al, Nature, 2001

Where does Suv39h1 act in a cell ???

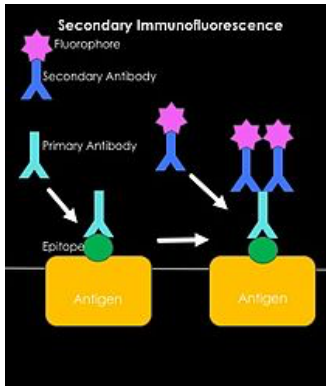
IMMUNOFLUORESCENCE → MACROSCOPIC ANALYSIS
→ Localization of protein across large regions of DNA



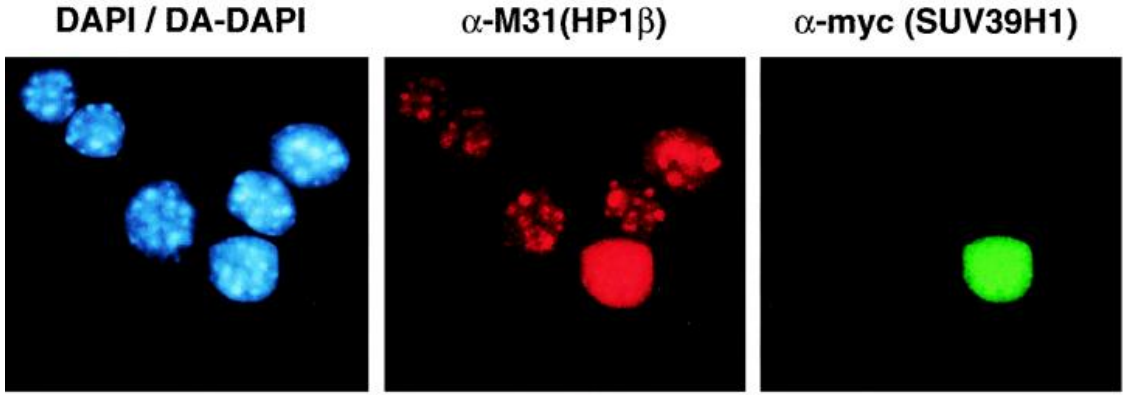
Loss of Suv39h1/h2 causes delocalization Of HP1 from chromocenters

Chromocenters are subnuclear regions where Multiple centromeres aggregate

Chromocenters are DAPI intense regions and represent tightly packed heterochromatin



Co-immunofluorescene (exicatiation with UV light):
Primary, mouse anti-HP1 + secondary rabbit anti-mouse-Cy3 (emission at 570 nm)
Primary, human anti-H3K9me3 + secondary donkey anti-human Alexa 488 (emission at 488 nm)
DAPI (emission 461 nm)

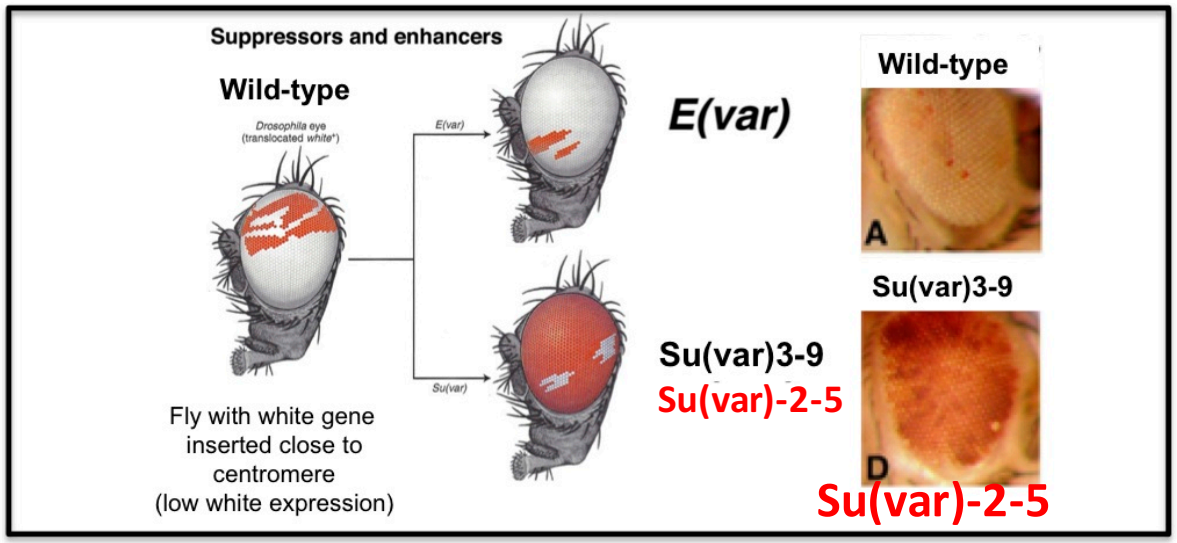


Over-expression of SUV39H1 in Hela cells causes an accumulation of HP1. Stabilization?
Is there a link between Suv39h1 – H3K9me3 and HP1??

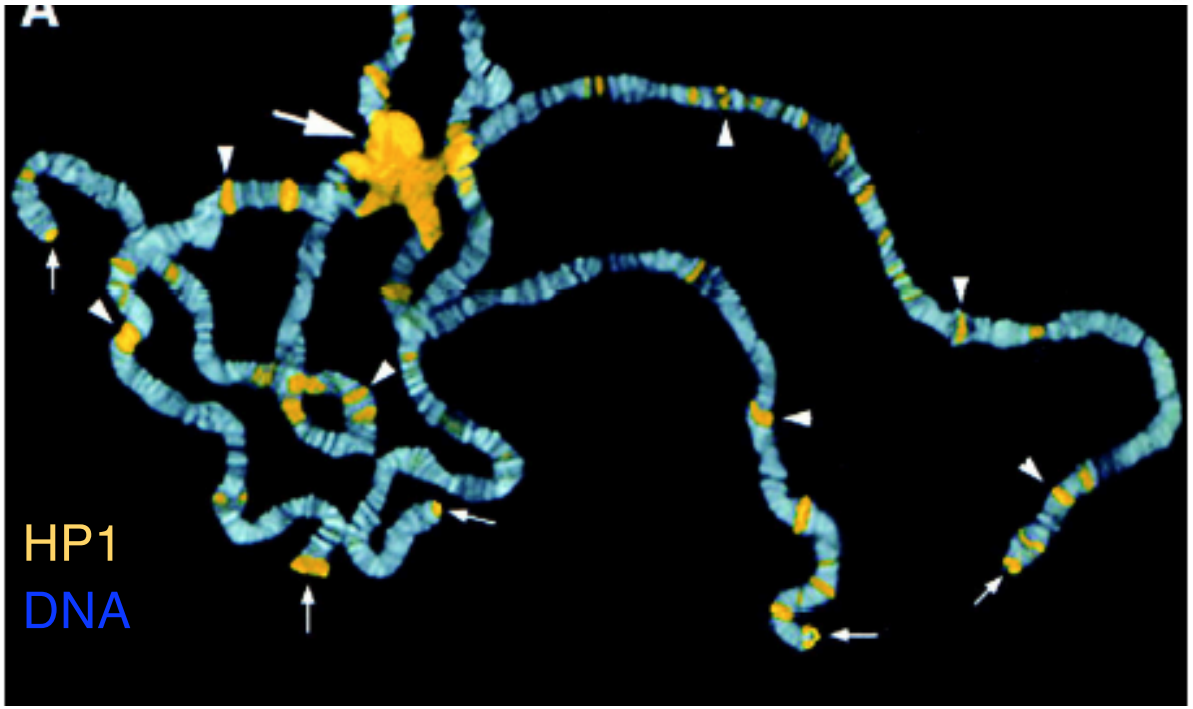
myc-SUV39H1: myc-epitope tagged Suv39H1

Melcher et al, MCB, 2000

What are the target sites for Suv39h1 and H3K9me???

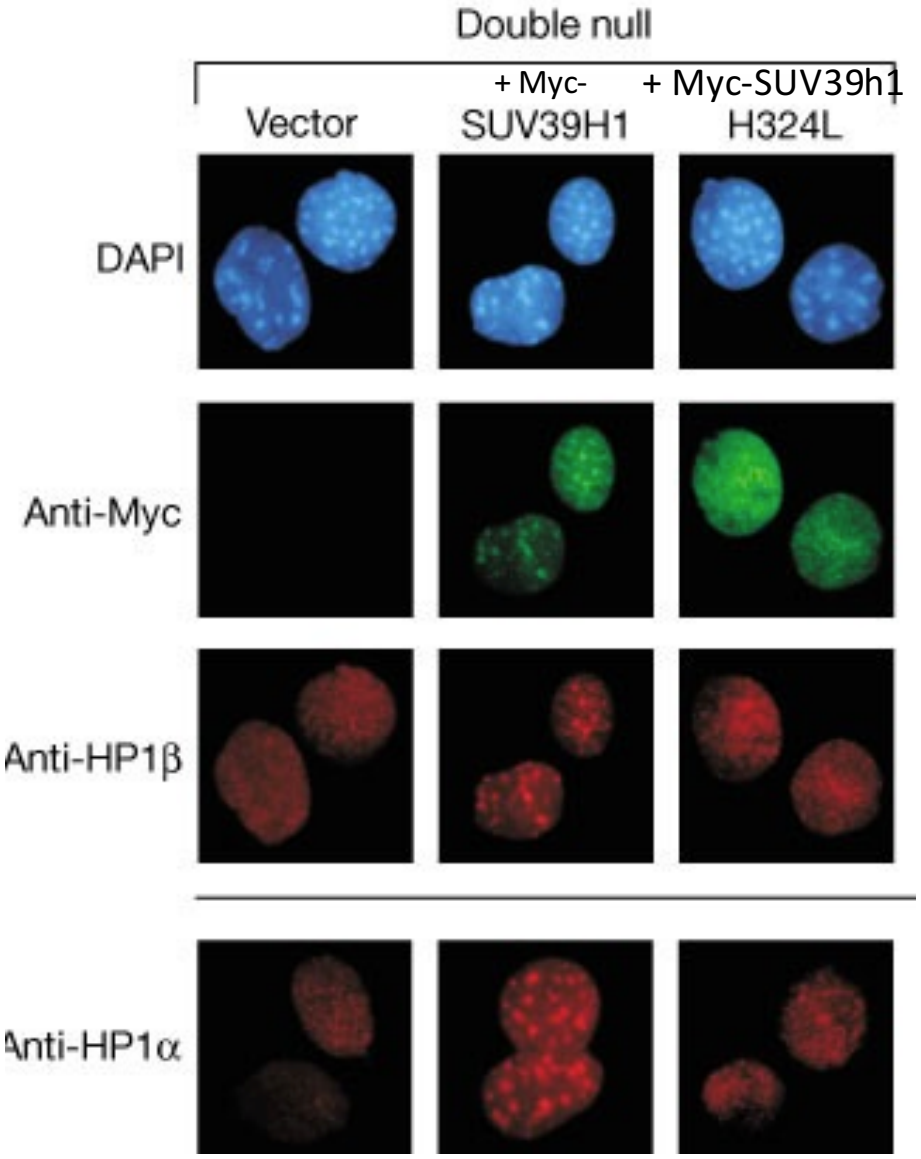


Another evidence for HP1 and Suv39h interaction came from Drosophlla



- back to early genetics studies in *Drosophila*:
- Su(var) 2-5 (gene) codes for heterochromatin protein 1
- Su(var) 2-5 shows similar phenotype like Su(var)3-9
- HP1 in Drosophila is localized to the chromocenter

SUV39h HMTase activity is important to build constitutive heterochromatin



Loss of Suv39h1 expression results in delocalization of HP1 from chromocenters

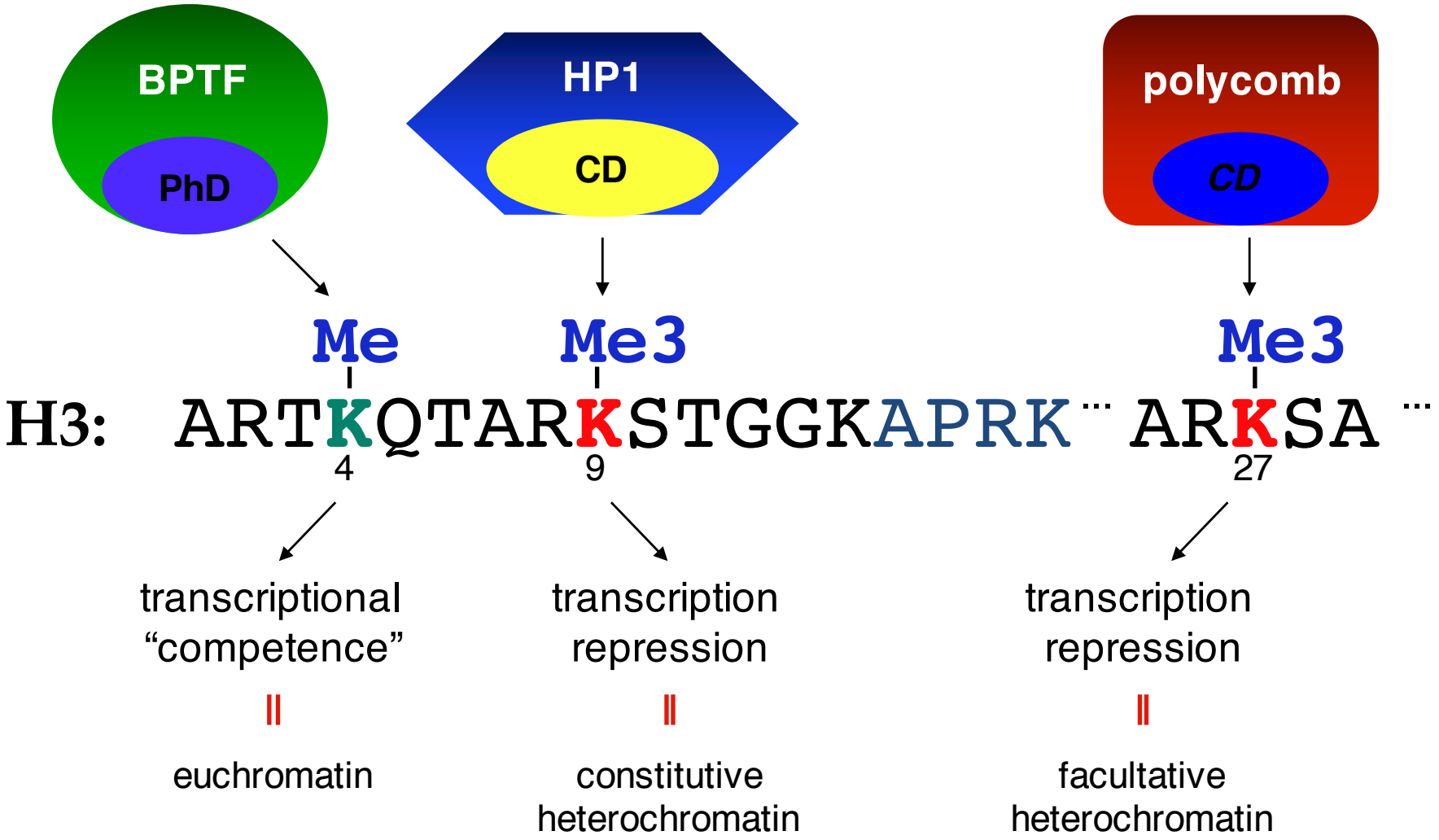
Re-expression of Suv39h1 rescues Localization of HP1 to chromocenters

Re-expression of Suv39h1 that Contains an enzymatic dead mutation (H324L) in SET domain does not rescue the localization of HP1 to chromo centers

→The enzymatic activity of Suv39h1 Is required for recruitment of HP1 to chromocenters

Lachner et al, Nature, 2001

Site specific methylation of the H3 tail has different functions

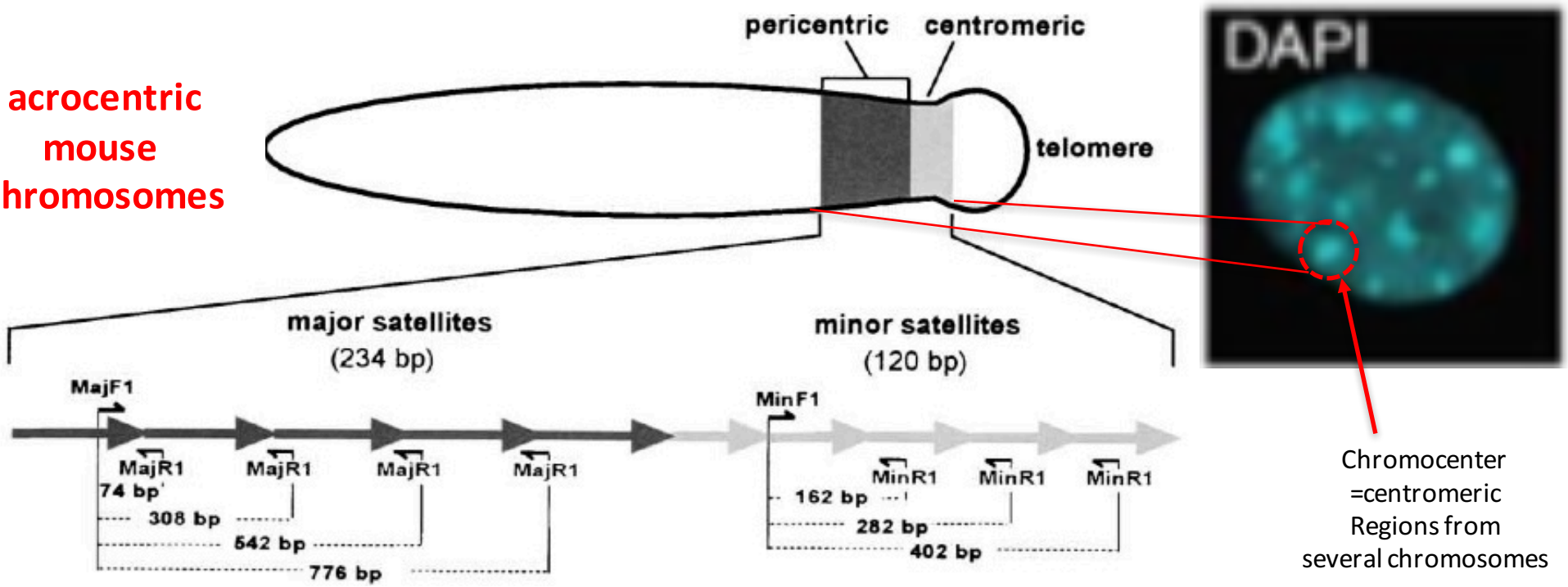


What are the target sites for Suv39h1 and H3K9me???

CHROMATIN IMMUNOPRECIPITATION → DETAILLED ANALYSIS
→ Localization of protein at a defined region - seqeunce

The combination of Immunoprecipitation methods and PCR analysis allows to define the histone code at defined sequences.
PCR primers define the site of analysis in the genome

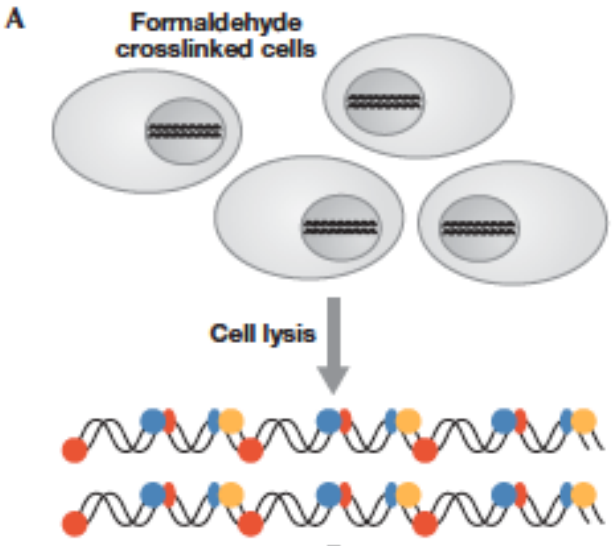
acrocentric mouse chromosomes



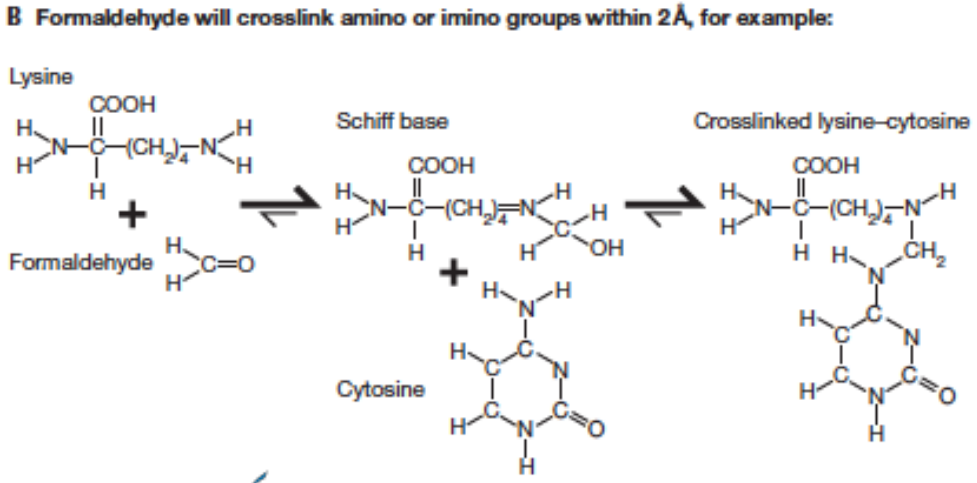
EXAMPLE: Pericentric heterochromatin in mouse cells

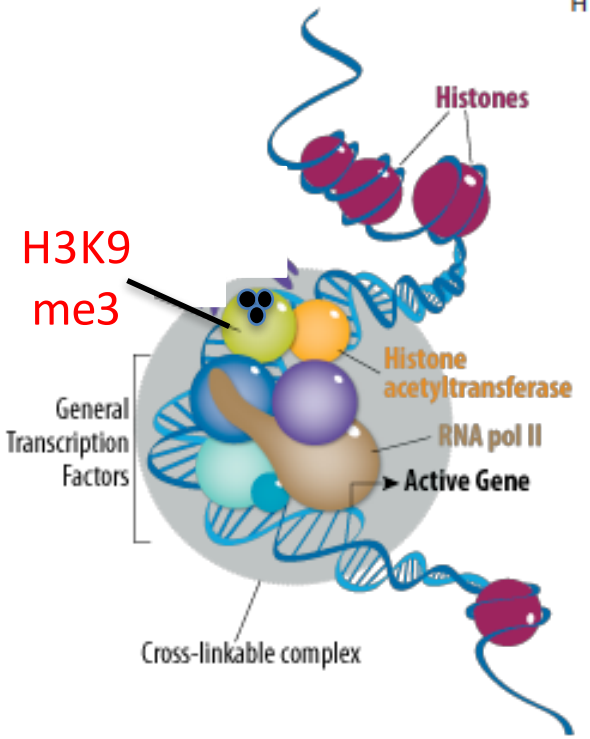
CHROMATIN IMMUNOPRECIPITATION

1. Cross linking with FA



Optimization is crucial.

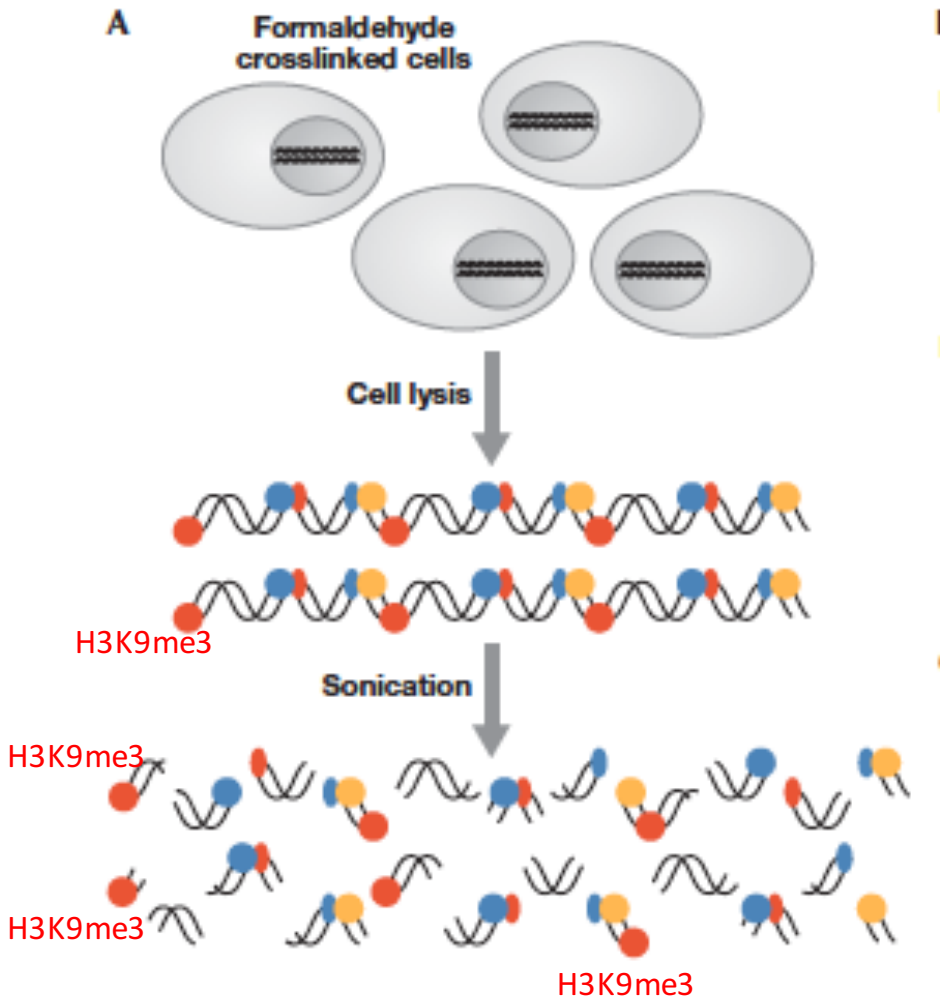


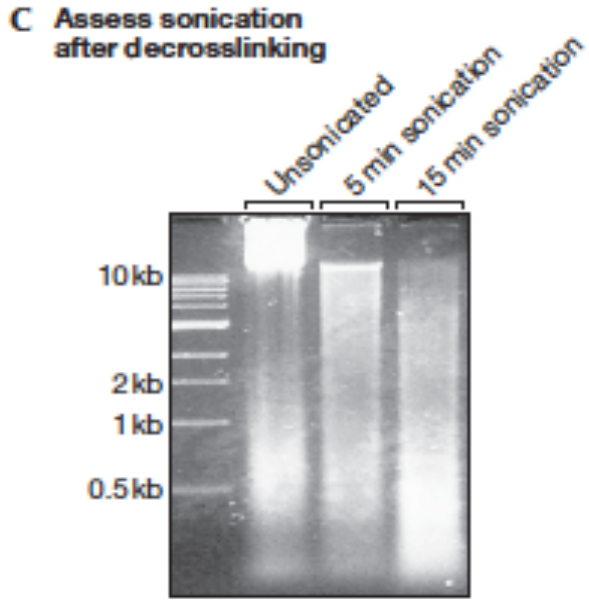


Covalently crosslinked chromatin

CHROMATIN IMMUNOPRECIPITATION

2. Cross linking followed by sonication (fragmentation of chromatin)



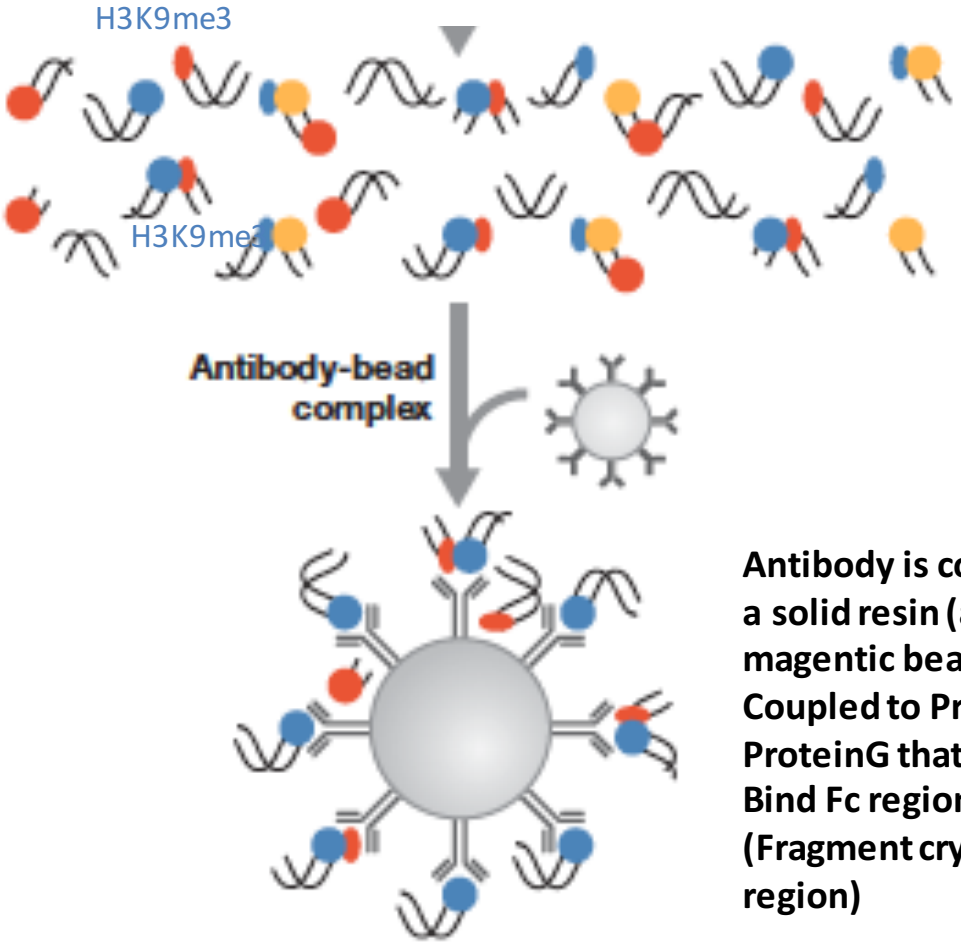


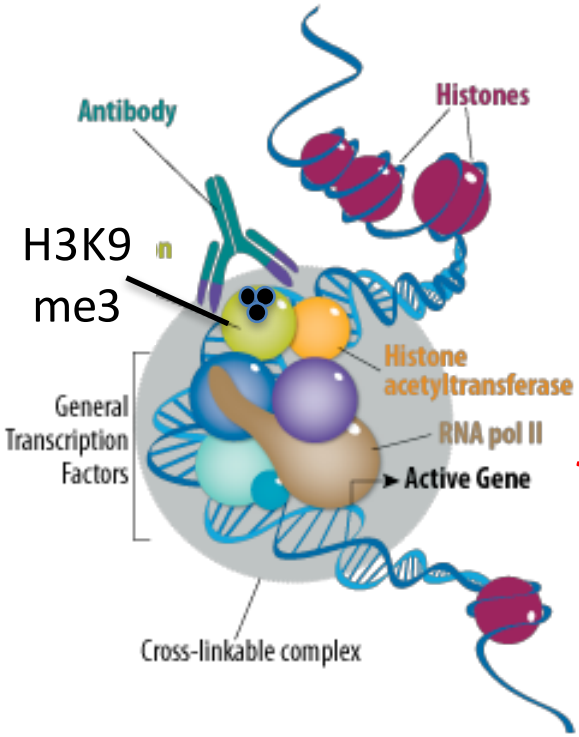
Chromatin is bulky → needs to be cut into small pieces to become soluble

CHROMATIN IMMUNOPRECIPITATION

3. Immunoprecipitation (IP)

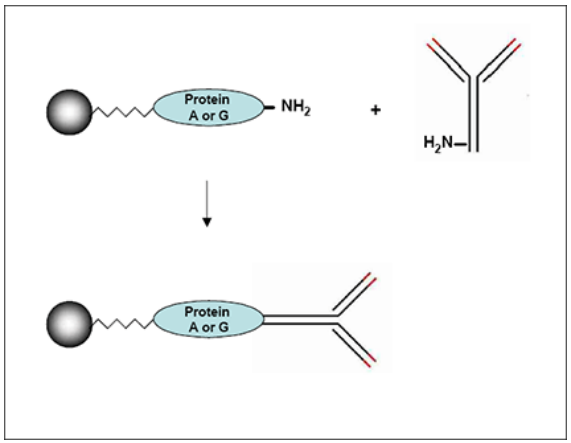
The protein of interest is immunoprecipitated together with the crosslinked DNA: Modified histones; epignetic writers, epigentic readers





Covalently crosslinked, fragmented Chromatin + Anti-H3K9me3

Antibody is coupled to a solid resin (agarose, magentic beads) Coupled to ProteinA or ProteinG that Bind Fc region of antibody (Fragment crystallizable region)



CHROMATIN IMMUNOPRECIPITATION

4.Decrosslinking of PFA crosslinked chromatin and and purification of the DNA

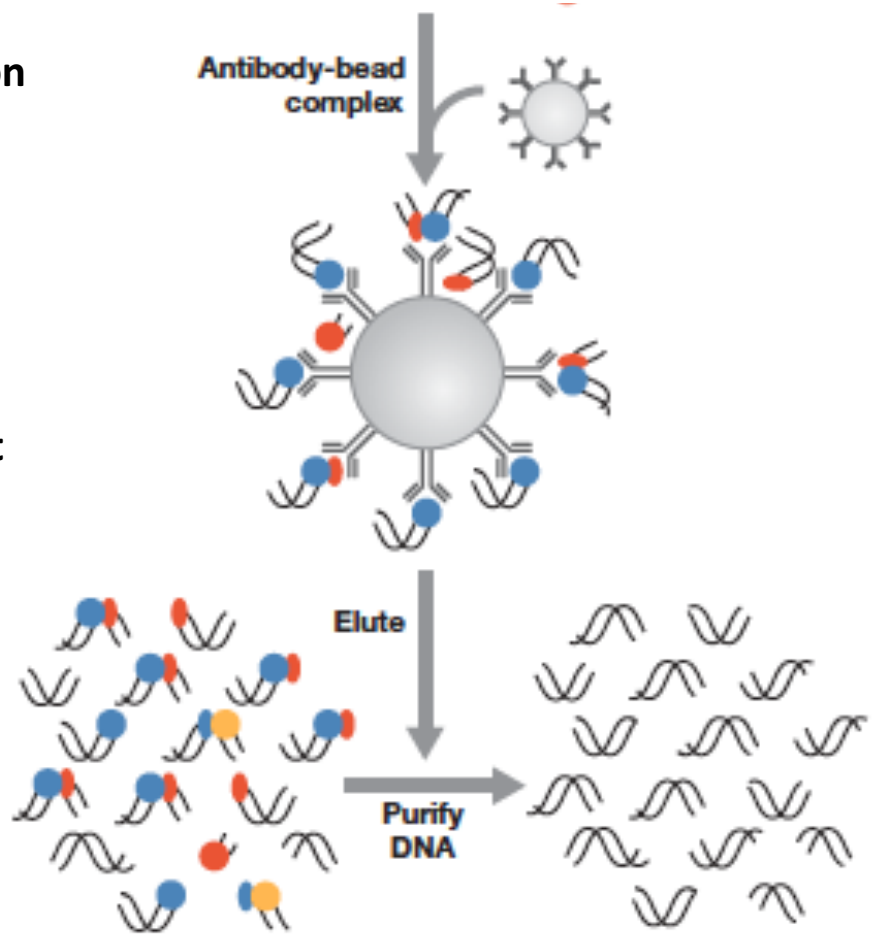
-Recover beads by centrofugation

- Wash beads

- Reverse the FA: 65°C, high salt concentrations: crosslink break

- RNAse and Protease treatment

- Purification of DNA

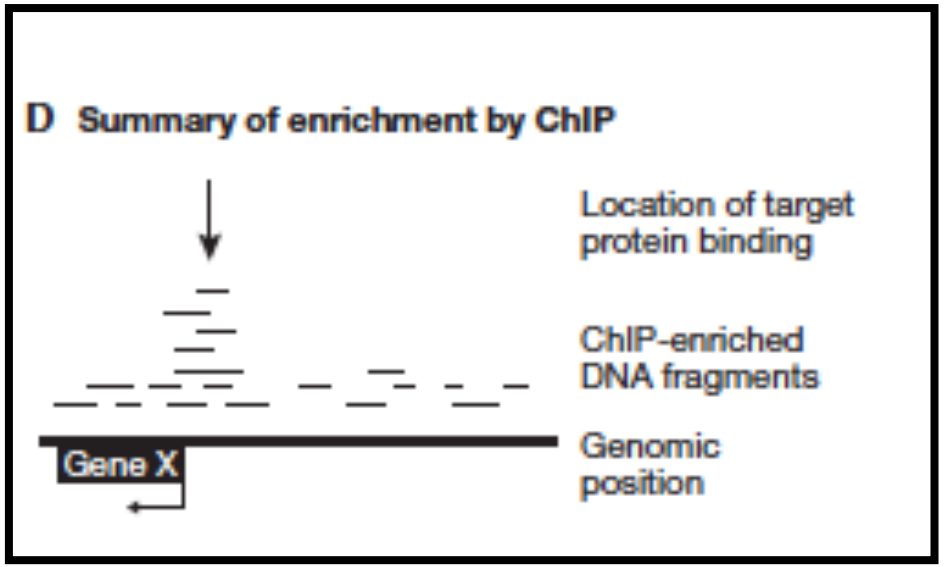


ANALYZE DNA TO IDENTIFY REGIONS WITH HIGH H3K9me3 (sequencing, hybridzation, PCR with gene specific primers

CHROMATIN IMMUNOPRECIPITATION

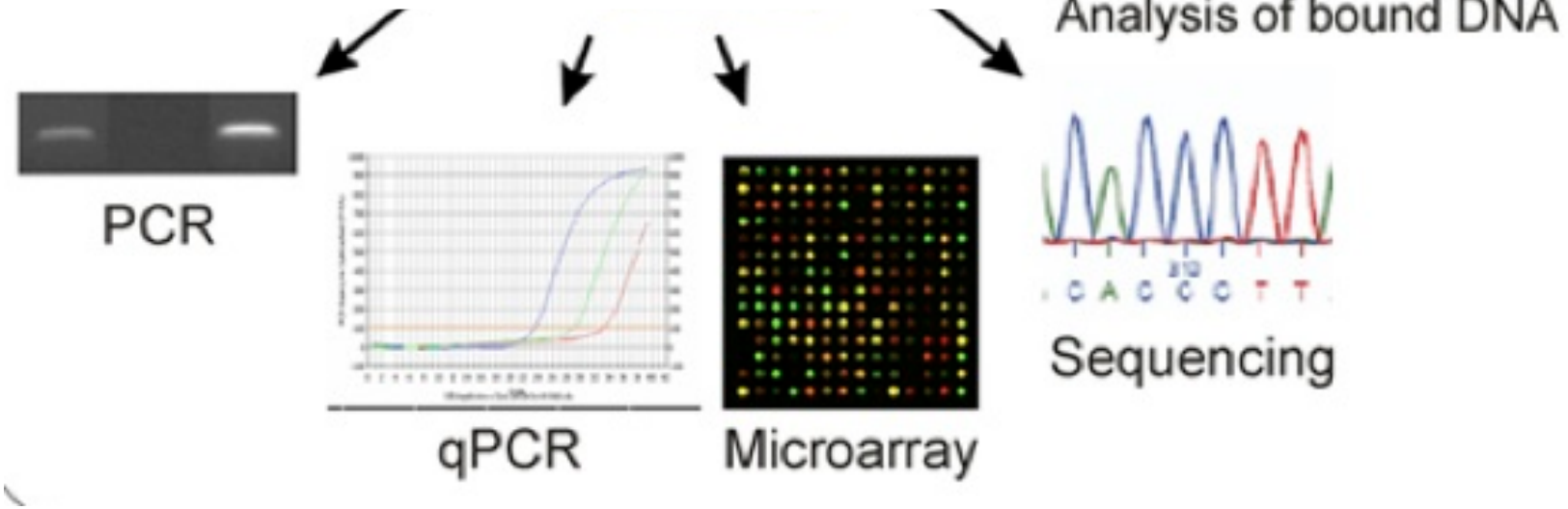
5. Analysis of ChIP DNA

Identification of DNA regions associated with the protein/modification of interest

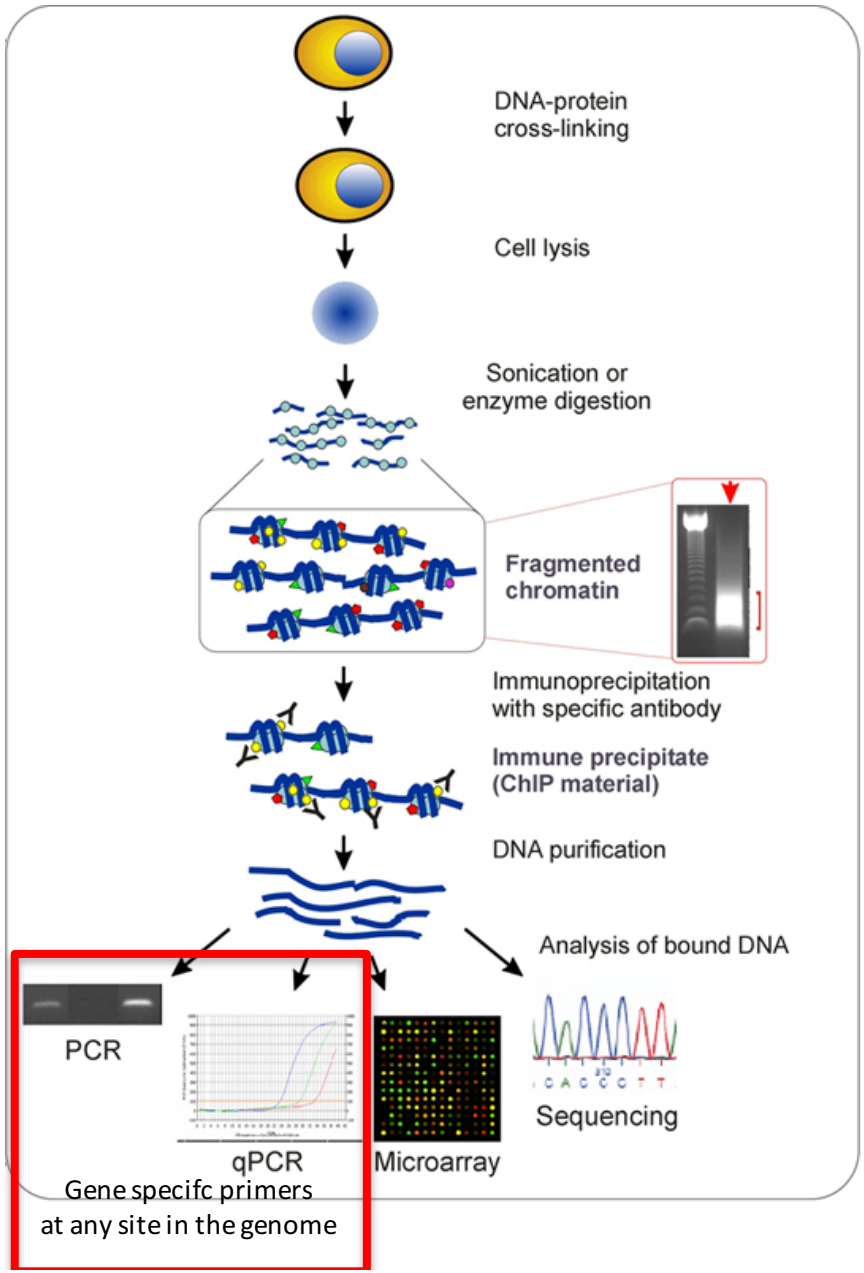


DNA from chromatin enriched in H3K9me
→ Correspond to precise sites in the genome

Methods of ChiP analysis



CHROMATIN IMMUNOPRECIPITATION (ChIP) → DETAILLED ANALYSIS
→ Localization of protein at a defined region - seqeunce



Cell model system:
i.e. Wild-type or Suv39 dn cells
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8. Measure the amount of immunoprecipitated DNA In control versus Suv39h dn cells

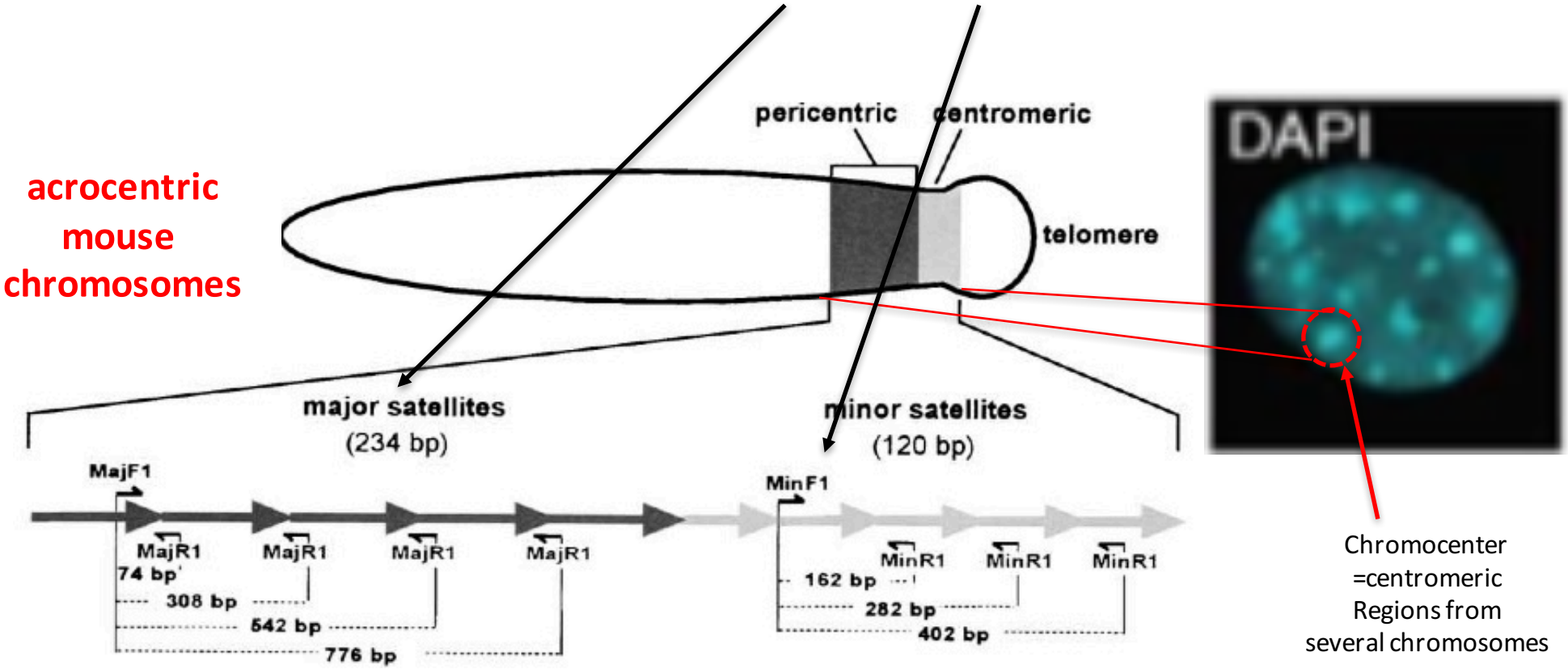
METHODS:

Quantitative PCR: design PCR oligos to amplify defined sequences in immunoprecipitates. Per PCR reaction only one Locus can be examined by real-time PCR

What are the target sites for Suv39h1 and H3K9me???

CHROMATIN IMMUNOPRECIPITATION FOLLOWED BY QUANTITTIVE PCR

Design PCR oligos that amplify major and minor satellite repeats

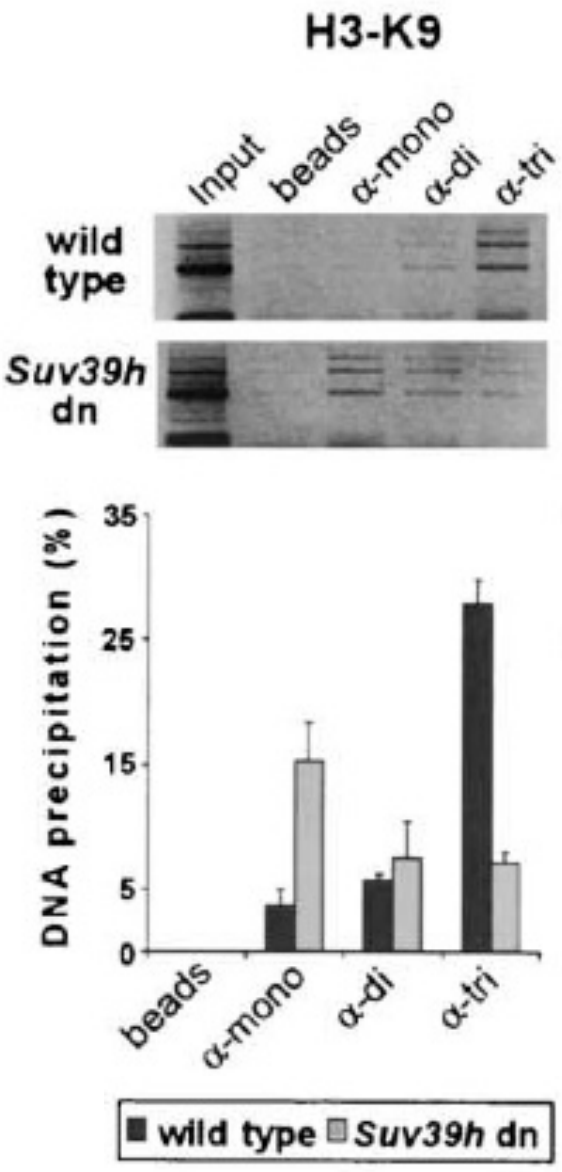


EXAMPLE: Pericentric heterochromatin in mouse cells

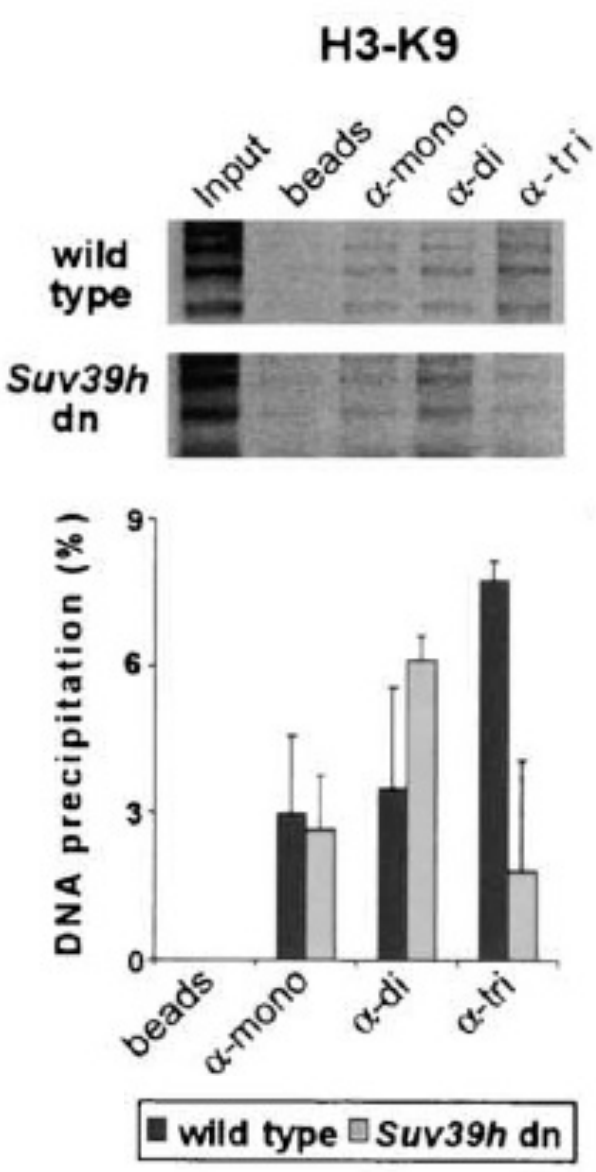
Min/Maj F1…: forward primer in unique region

CHROMATIN IMMUNOPRECIPITATION (ChIP) COUPLED WITH PCR
→ H3K9me3 is enriched at pericentric (major+minor) repeats in mouse cells

Major satellite repeats



Minor satellite repeats

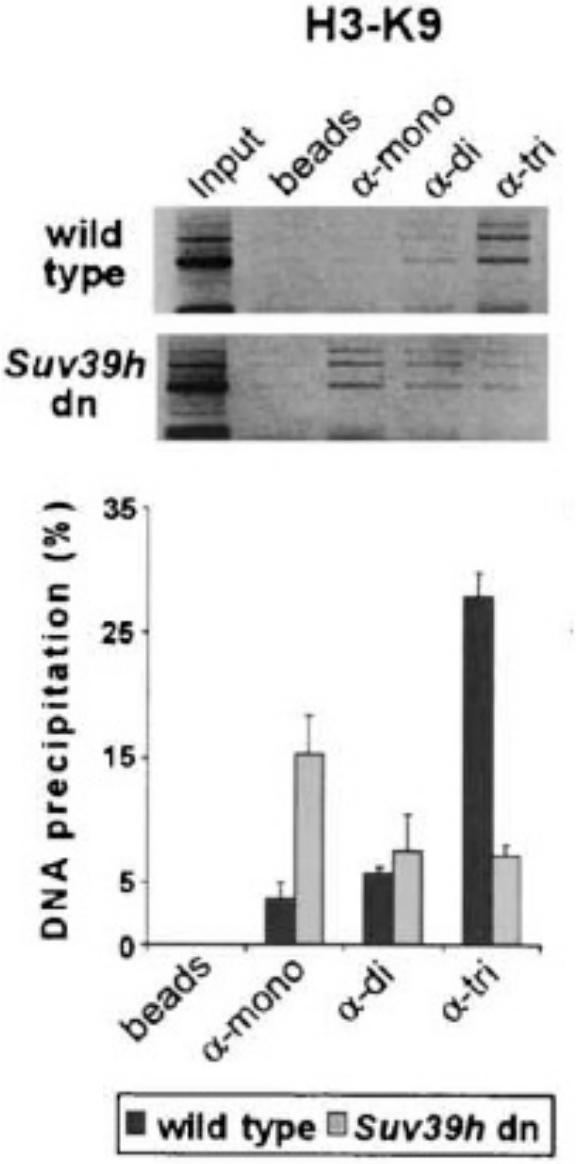


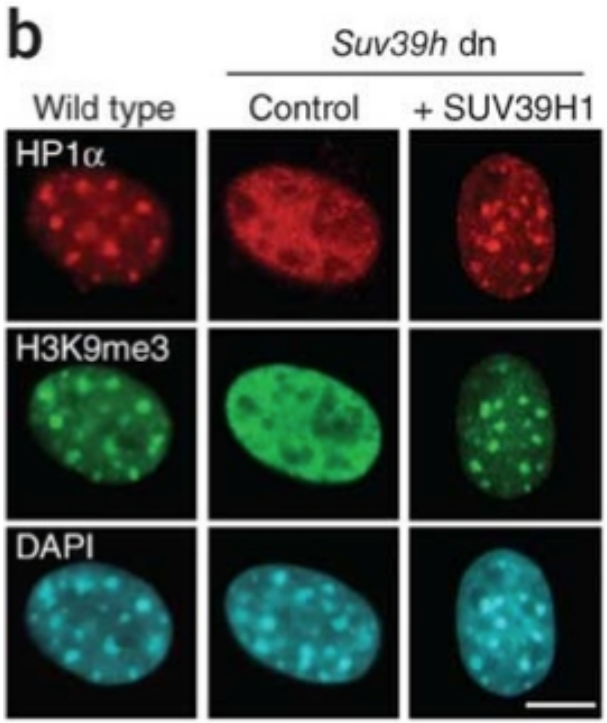
PCR amplification of major/minor satellite Repeats after ChiP using Antibodies that are specific for H3K9me1; H3K9me3; H3K9me3

Suv39h1 is required for imposition of H3K9me3 at pericentric repeats

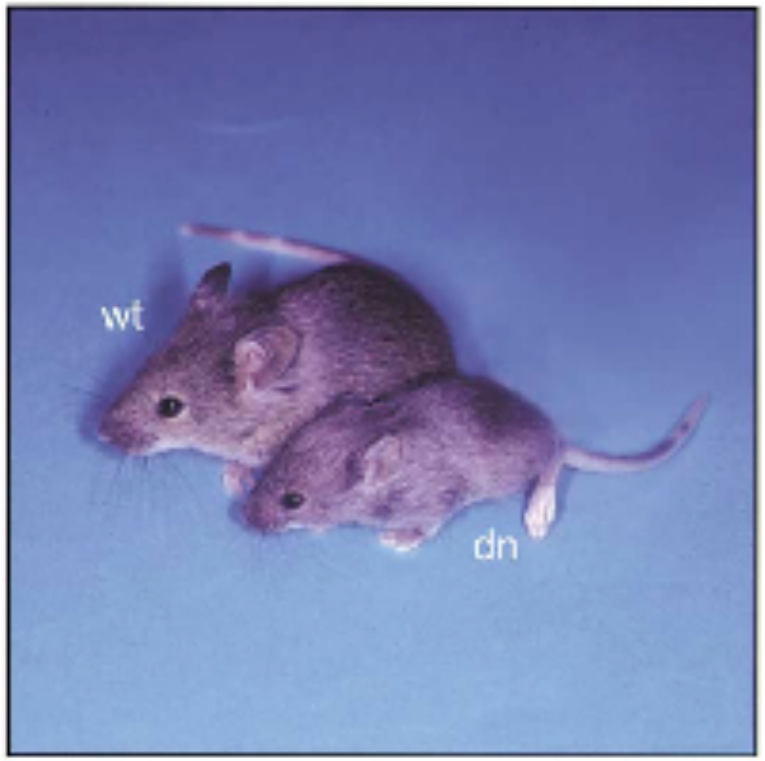
From molecular data to phenotypes and biological relevance

Major satellite repeats



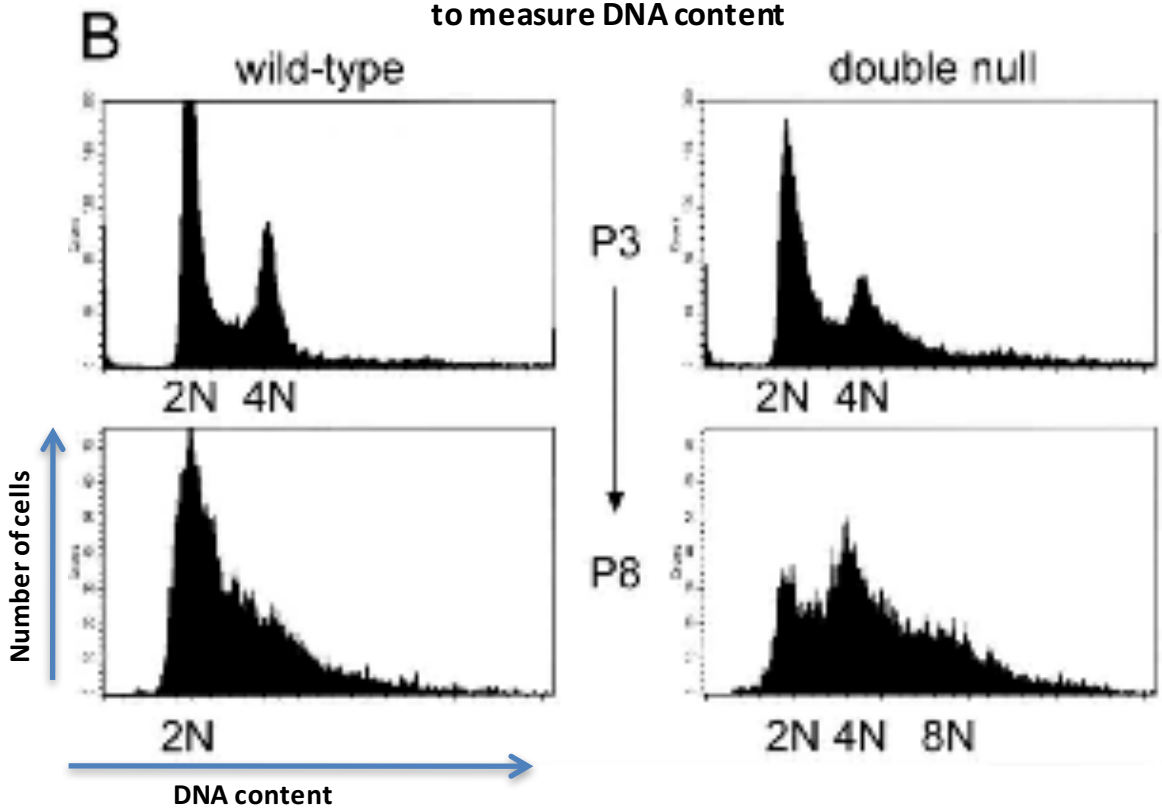


Lack of SUV39h HMTase activity results in genomic instability



A knock-out model system
for Suv39h1 and Suv39h2
- Loss of Suv39h1/2: smaller body size

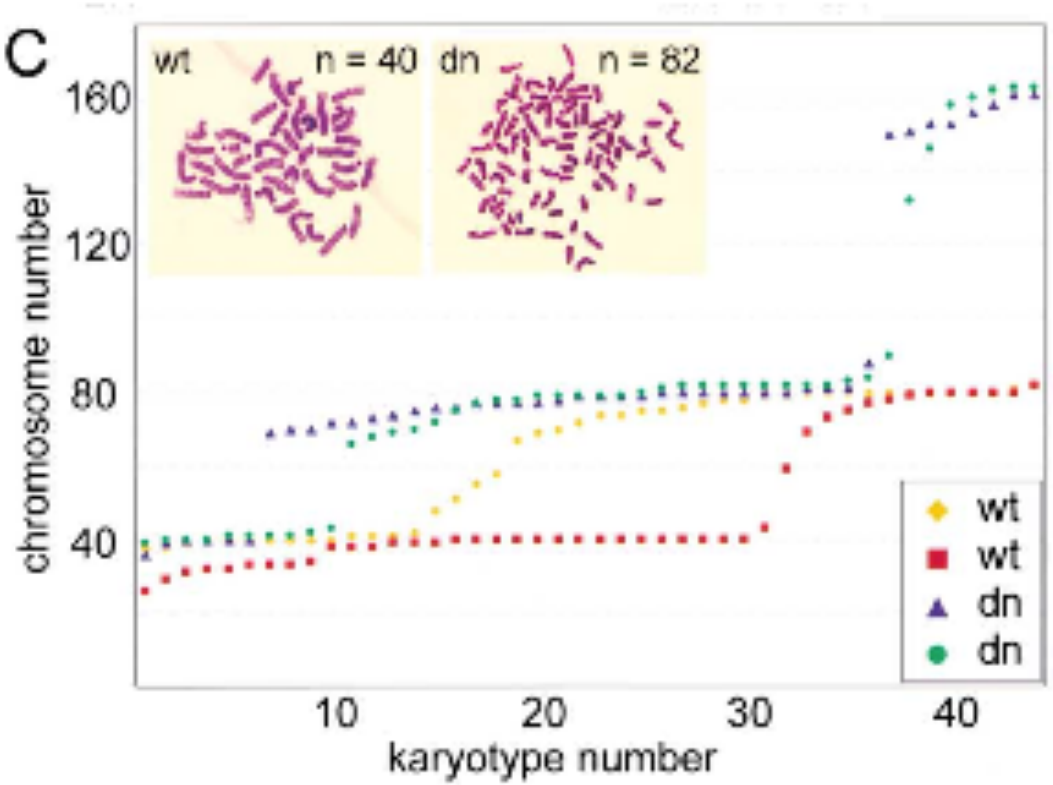
Fluorescence activated cell sorting (FACS)
to measure DNA content



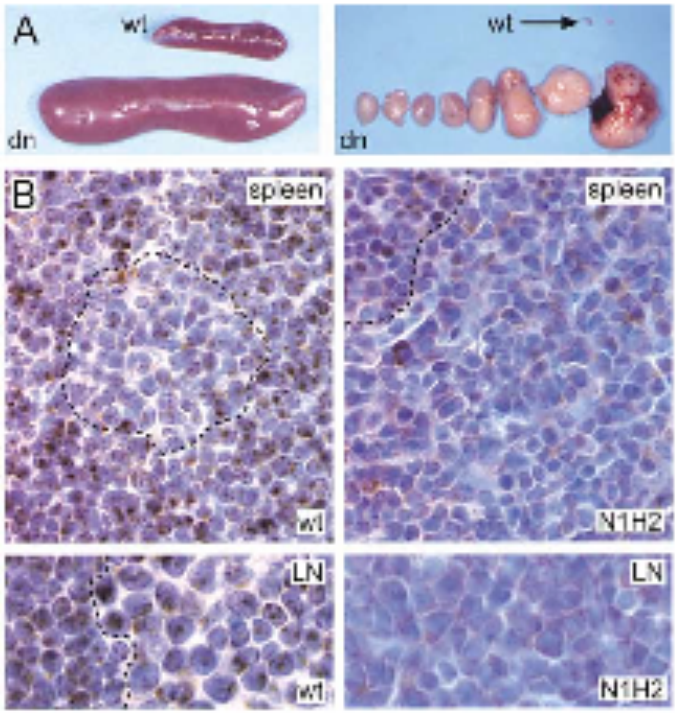
Fibroblasts from Suv39h1/2 null mice
are aneuploidy

Lack of SUV39h HMTase activity results in genomic instability

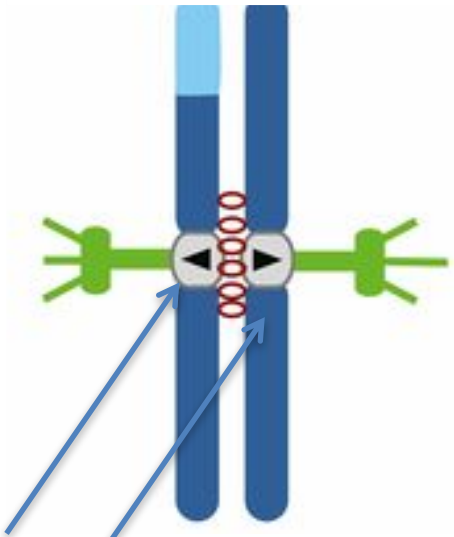
Loss of Suv39h1/2 results in increased chromosome numbers



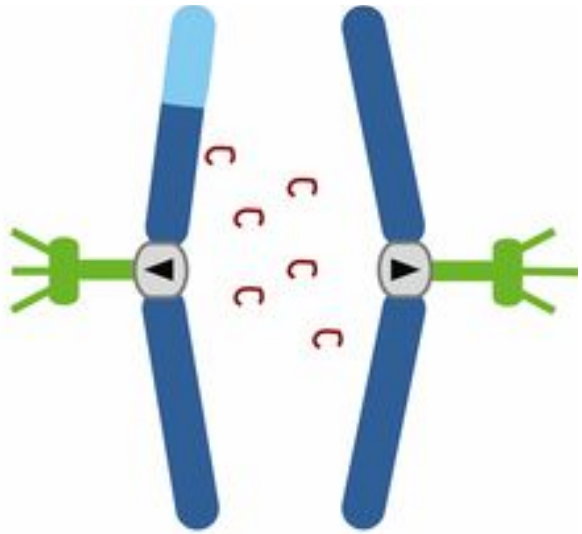
Genomic instability in Suv39h1/2 mice increases lymphomas



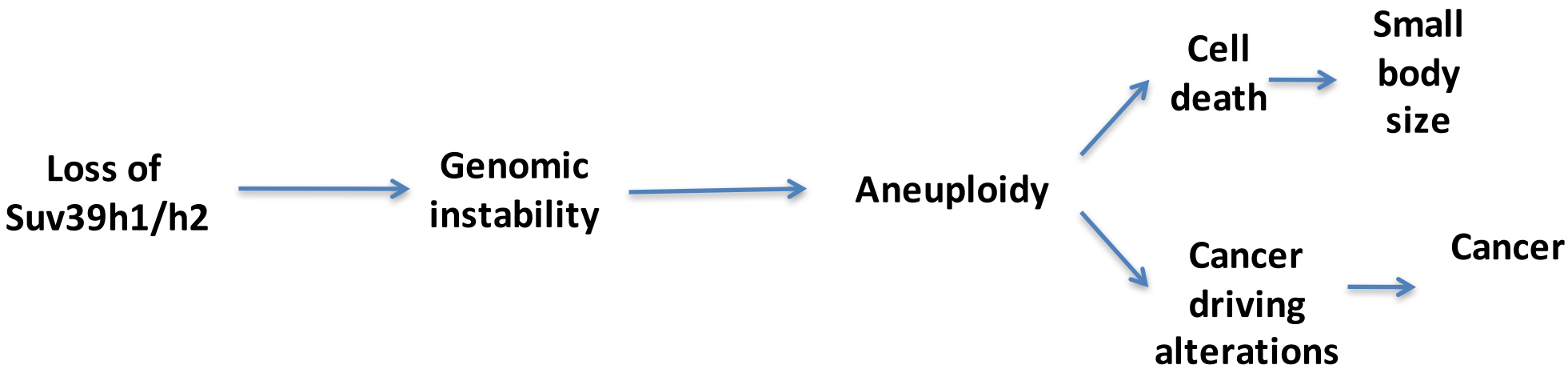
SUV39h HMTase activity is essential for fidelity in mitosis/meiosis



perientric heterochomatin



Correct heterochromatin structure supports chromosome segregation by the mitotic/meiotic spindle apparatus



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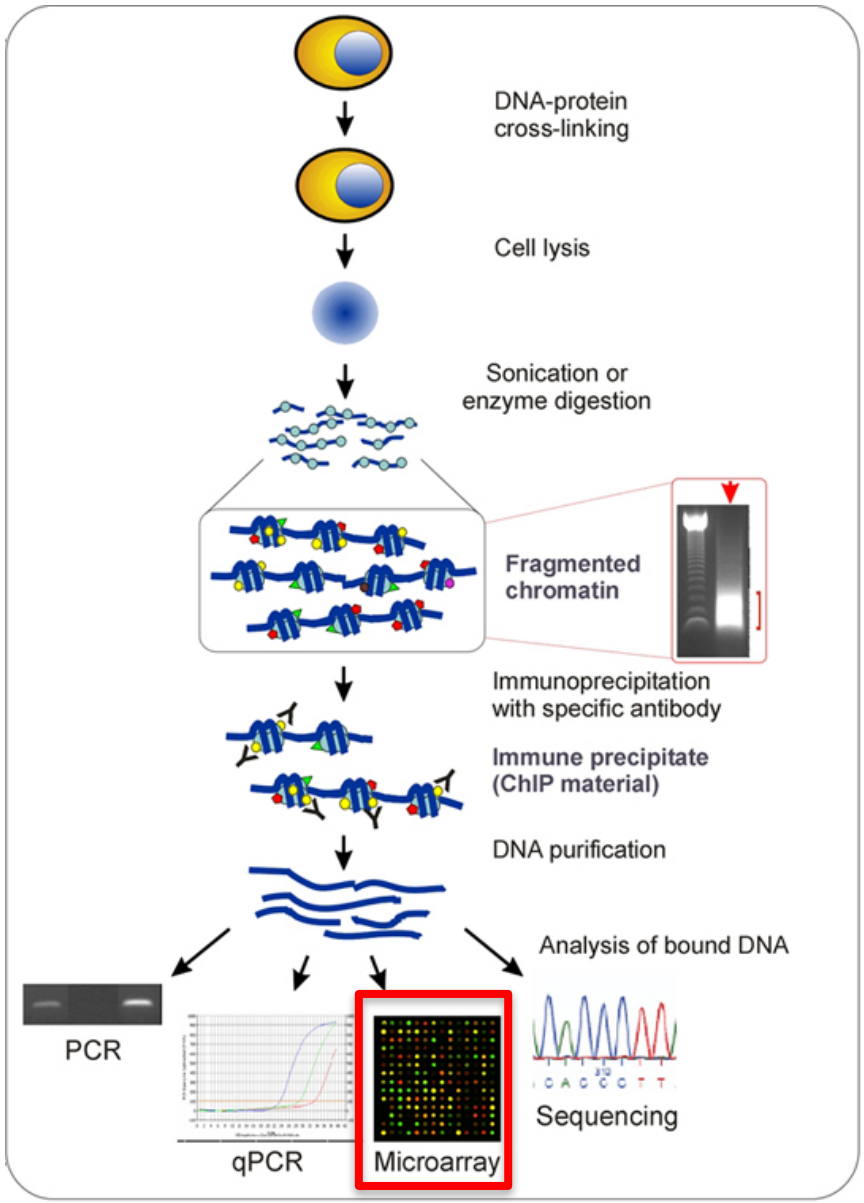

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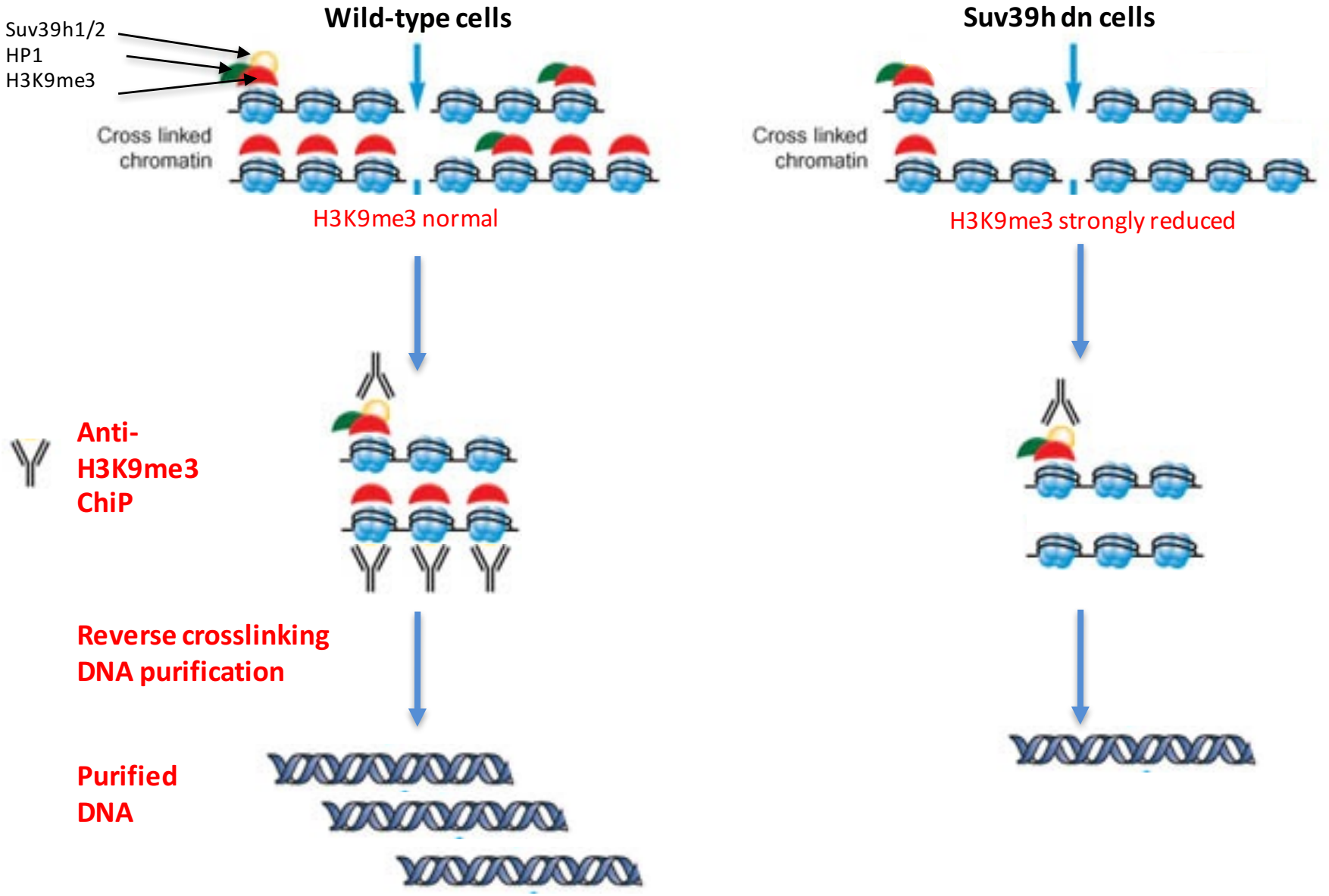
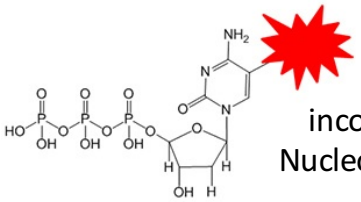
ChIP on ChIP: Analysis of epigenetic information across a high number of genomic sites in the genome



ChIP on ChIP: Analysis of epigenetic information across a high number of genomic sites in the genome

Purified DNA From ChIP

Wild-type cells

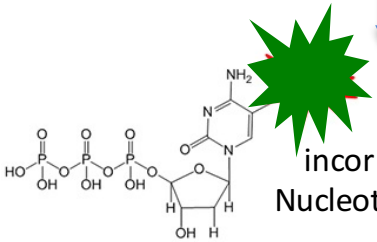


Denaturalization of DNA;
priming of DNA synthesis;
incorporation of fluorescently labeled
Nucleotides into newly synthesized strand

Fluorescently labeled probe

Cy5- labeled
(~650 excitation, 670 nm emission)

Suv39h dn cells

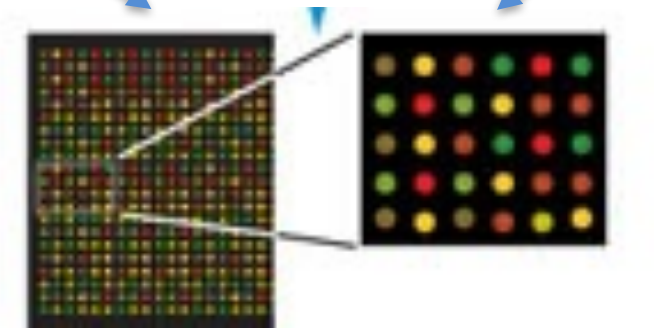


Denaturalization of DNA;
priming of DNA synthesis;
incorporation of fluorescently labeled
Nucleotides into newly synthesized strand

Cy3- labeled
(550 nm excitation, ~570 nm emission)

Mix probes

Hybridize
to a genome array
= dots: spotted short DNA probes that represent defined regions of a genome (promoters, gene bodies, exons…). Each defined region is represented by multiple spots



Measure Green/Red ratio

ChIP on ChIP: Analysis of epigenetic information across a high number of genomic sites in the genome

Purified DNA From ChIP

Wild-type cells

Suv39h dn cells

Fluorescently labeled probe

Cy5- labeled
(~650 excitation, 670 nm emission)

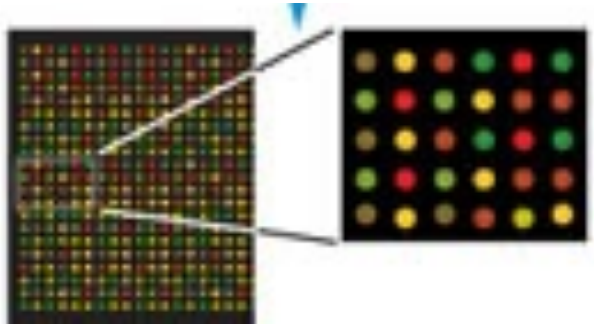
Cy3- labeled
(550 nm excitation, ~570 nm emission)

Site in genome	labelled probe WT	labelled probe Suv39dn	Signal on array
1. H3K9me3 (by Suv39h)			
2. H3K9me3 (not by Suv39h)			
3. No H3K9me3			

Intesity

high medium low Quantitative info

Mix probes

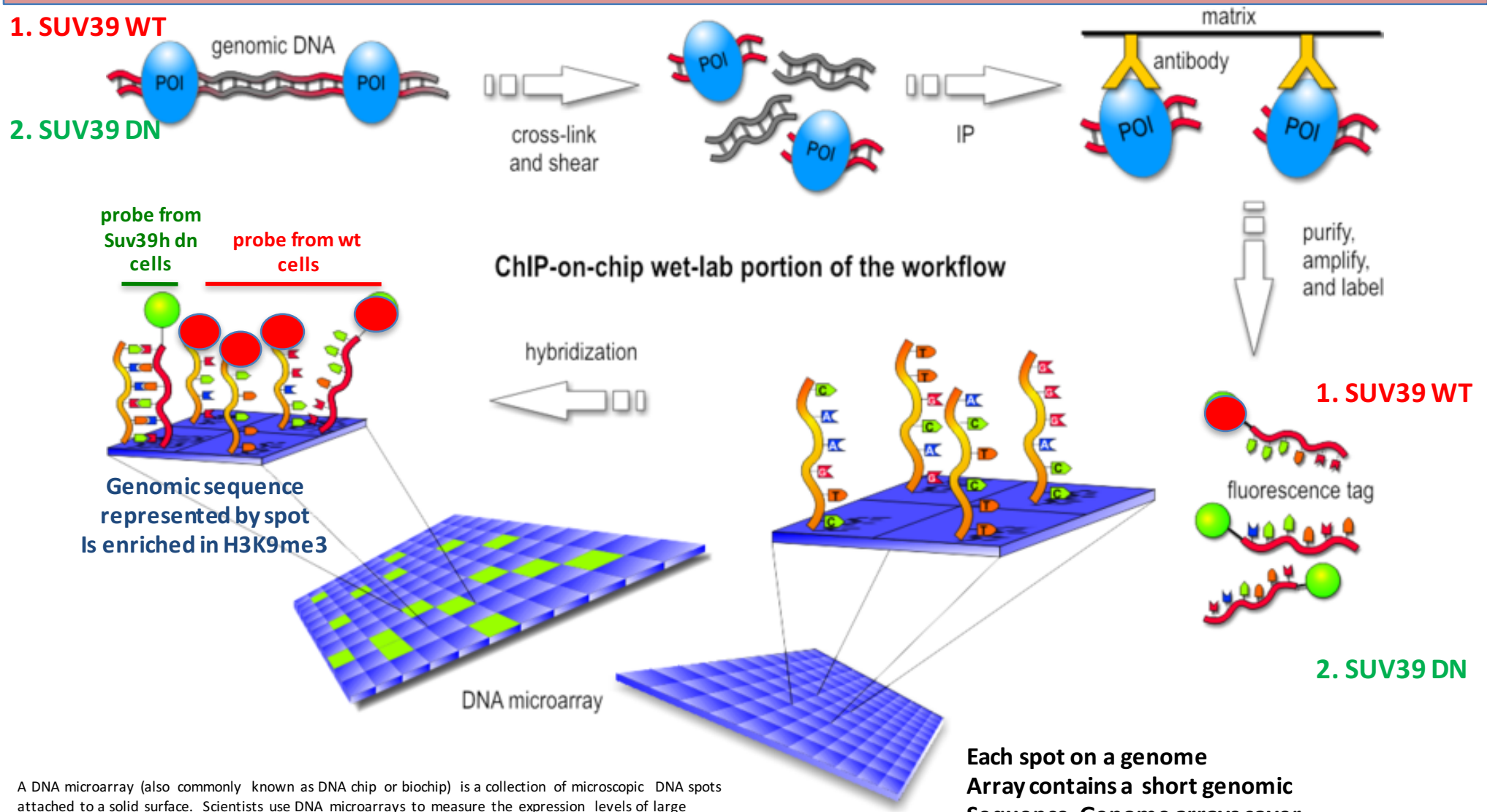


Measure Green/Red ratio
Hybridize to a genome array

Scan:

1. *Red channel (+normalize signal across array)*
2. *Green channel (+normalize signal across array)*

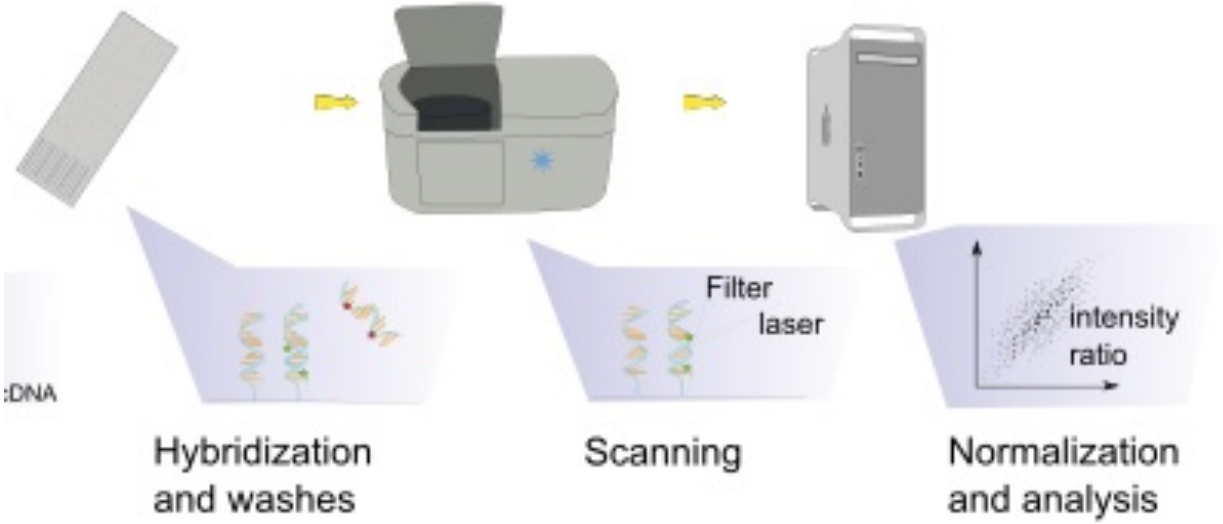
ChIP on ChIP: Analysis of epigenetic information across a high number of genomic sites in the genome

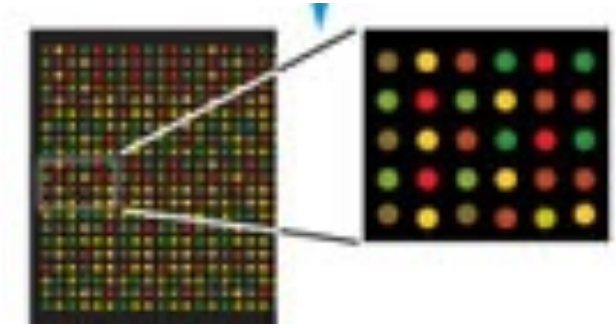


A DNA microarray (also commonly known as DNA chip or biochip) is a collection of microscopic DNA spots attached to a solid surface. Scientists use DNA microarrays to measure the expression levels of large numbers of genes simultaneously or to genotype multiple regions of a genome. Each DNA spot contains picomoles (10−12 moles) of a specific DNA sequence, known as probes (or reporters or oligos). These can be a short section of a gene or other DNA element that are used to hybridize a cDNA or cRNA (also called anti-sense RNA) sample (called target) under high-stringency conditions. Probe-target hybridization is usually detected and quantified by detection of fluorophore-, silver-, or chemiluminescence-labeled targets to determine relative abundance of nucleic acid sequences in the target

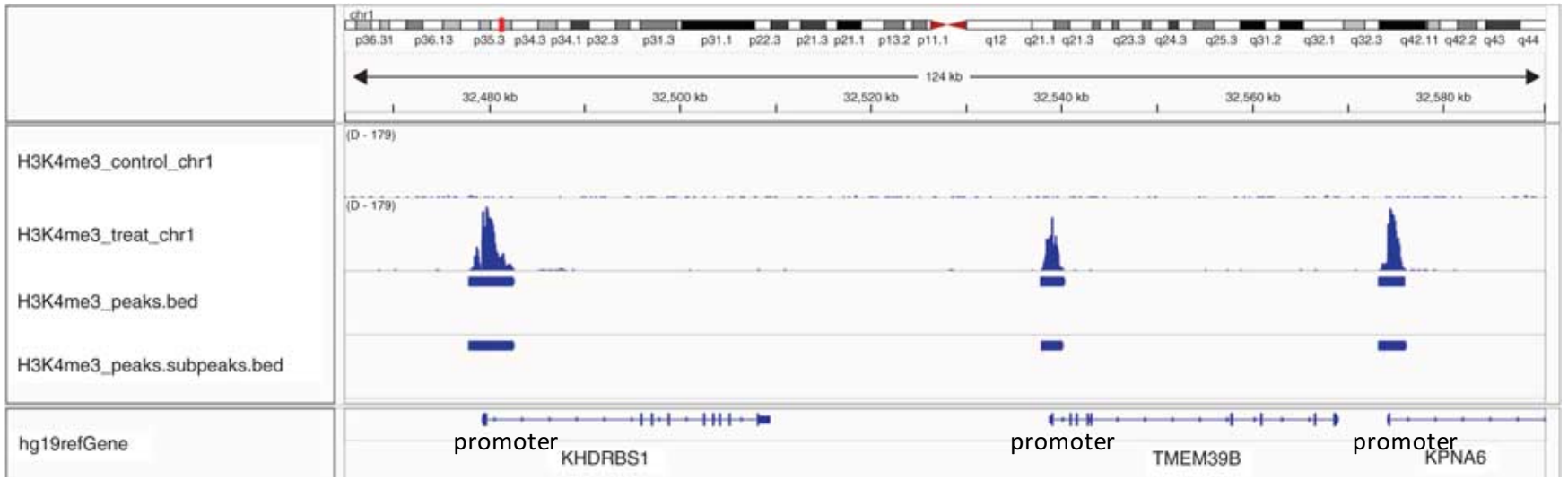
Each spot on a genome Array contains a short genomic Sequence. Genome arrays cover a large part of the Genome
A ChIP holds ca 20000 spots

ChIP on ChIP: Analysis of epigenetic information across a high number of genomic sites in the genome





Arrays do not contain the entire human/mouse genome
Arrays are enriched for particular seqeunces according to experimental need (promoter, enhancer, etc…)



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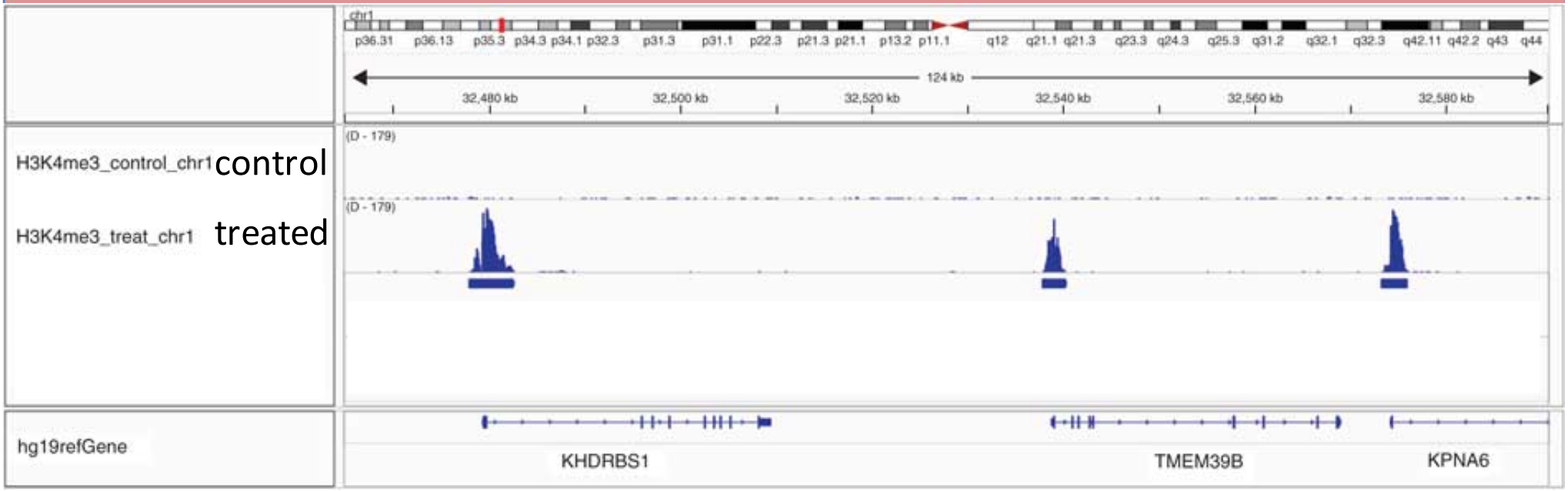
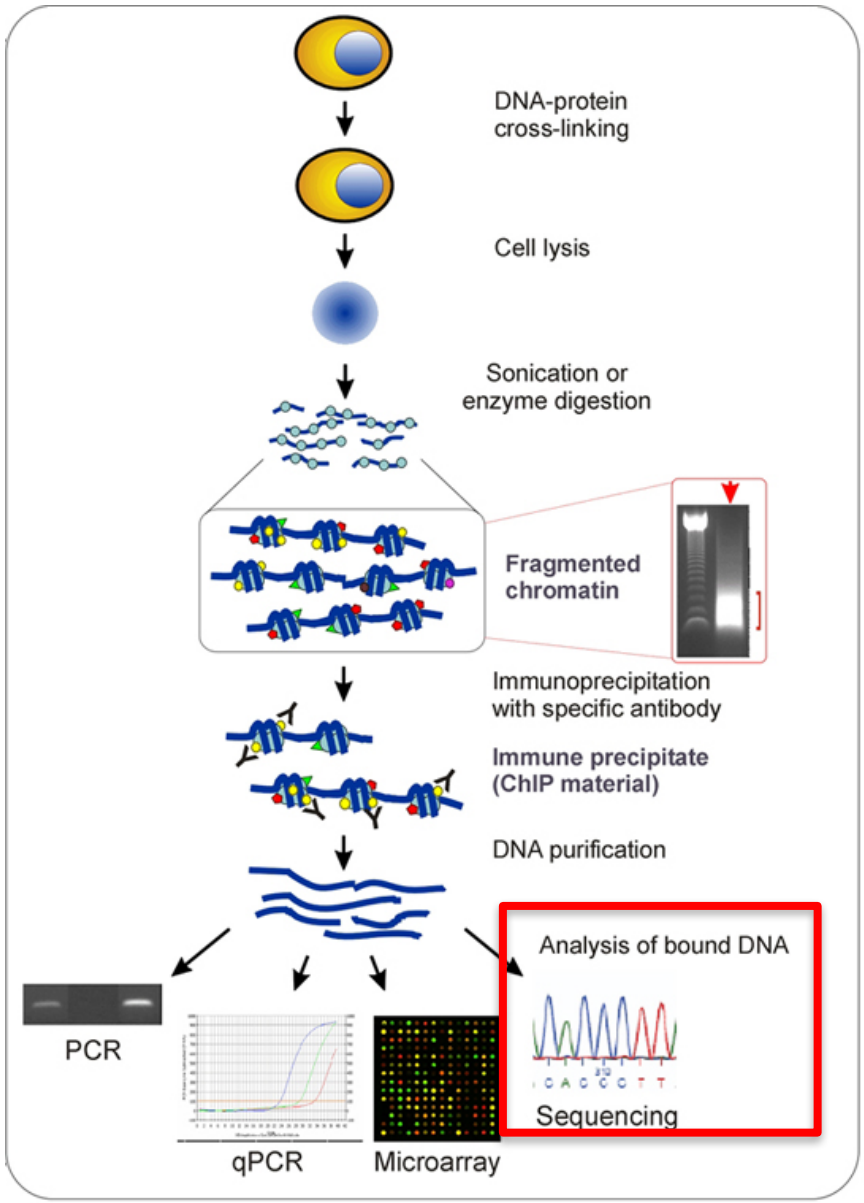


Advantage: low tech, cheap

Disadvantage: low resolution, no data on number of molecules – just proportions; laborious to reach a good genome coverage

Already outdated → state of the art: ChIP seq

ChIP seq: Analysis of epigenetic information on the single nucleotide level
→ GENERATION OF GENOME WIDE EPIGENTIC MAPS



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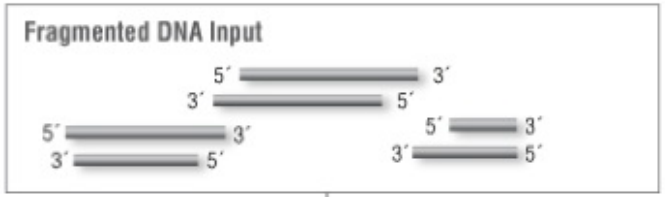
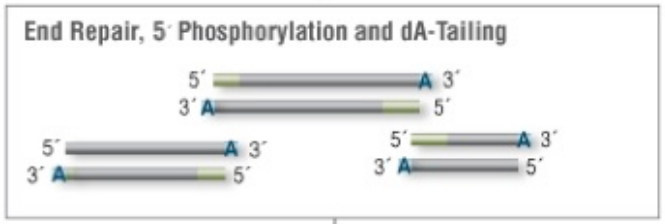
METHODS:

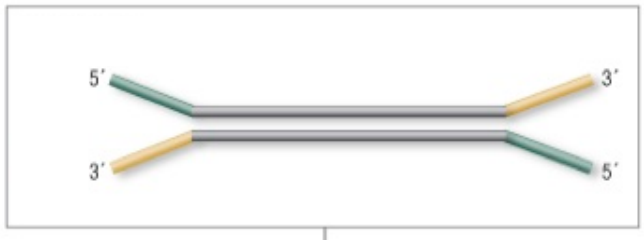
Massive parallel sequencing of immunoprecipitated DNA
Permits to obtain epigenetic information on the single nucleotide level

ChIP seq: Analysis of epigenetic information on the single nucleotide level
→ GENERASTION OF GENOME WIDE EPIGENTIC MAPS

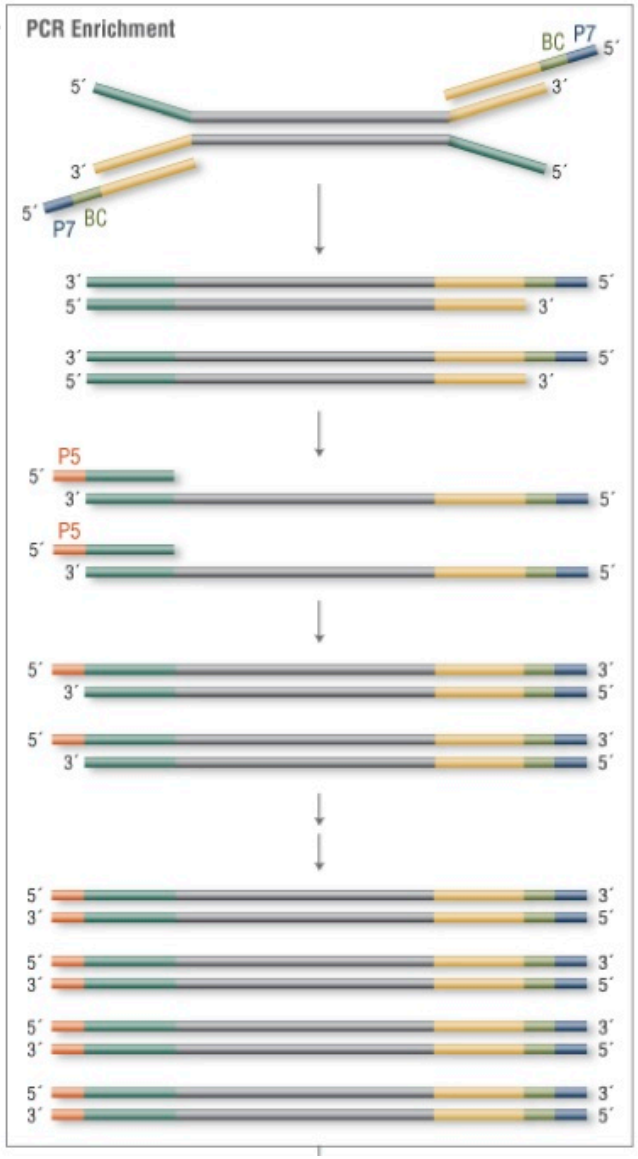
Use immunoprecipitated DNA to generate DNA libraries:







fusion of DNA with linker oligo



Linkers serve as uniform primer binding sites. This allows the amplification of the entire DNA library using only 2 types of oligonucleotides

Amplified library

READY FOR MASSIVE PARALLEL SEQEUNCING

ChIP seq: Analysis of epigenetic information on the single nucleotide level
→ GENERASTION OF GENOME WIDE EPIGENTIC MAPS

Illumina Massively Parallel Sequencing

https://www.illumina.com/company/video-hub/pfZp5Vgsbw0.html

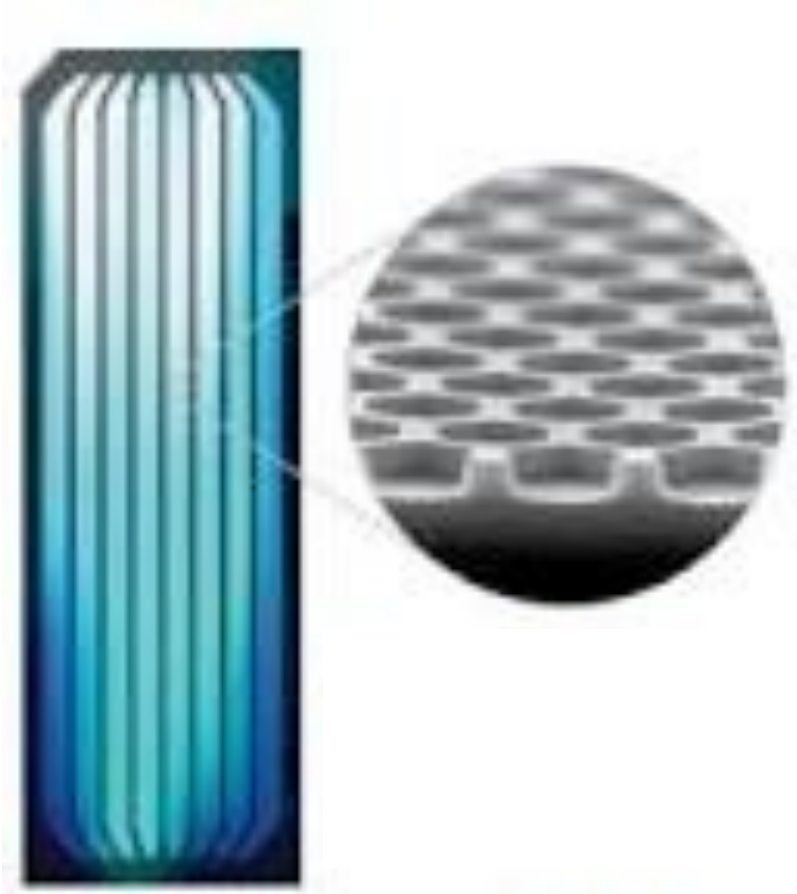
The heart of the Illumina Massive Parallel Sequencer is the "FLOW-CELL". A surface with millions of small wells that allow thousands of Sanger-sequencing reaction In parallel = "massive parallel sequencing". In each well a SINGLE MOLECULE of DNA Is amplified and sequenced

Illumina offers the most potent massive sequencing instruments – leader on the market

https://www.youtube.com/watch?v=pfZp5Vgsbw0

ChIP seq: Analysis of epigenetic information on the single nucleotide level
→ GENERASTION OF GENOME WIDE EPIGENTIC MAPS

CLUSTER AMPLIFICATION:



Flow cell contains surface with millions of wells

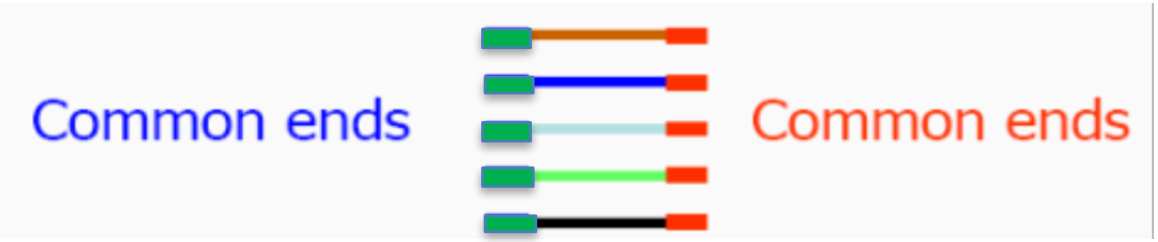
→Each well contains beads mounted with 2 species of oligonucleotides that hybridize with adaptor oligos of DNA library

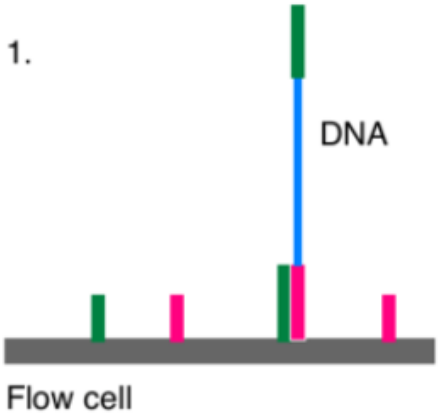
→DNA library will be loaded onto the flow cell in a determined concentration:

ONLY ONE MOLECULE OF DNA WILL BE PROCESSED FOR SEQUENCING IN A SINGLE WELL

CLUSTER AMPLIFICATION:

-making DNA library (~300bp fragments)
-ligation of adapters **A** and **B** to the fragments





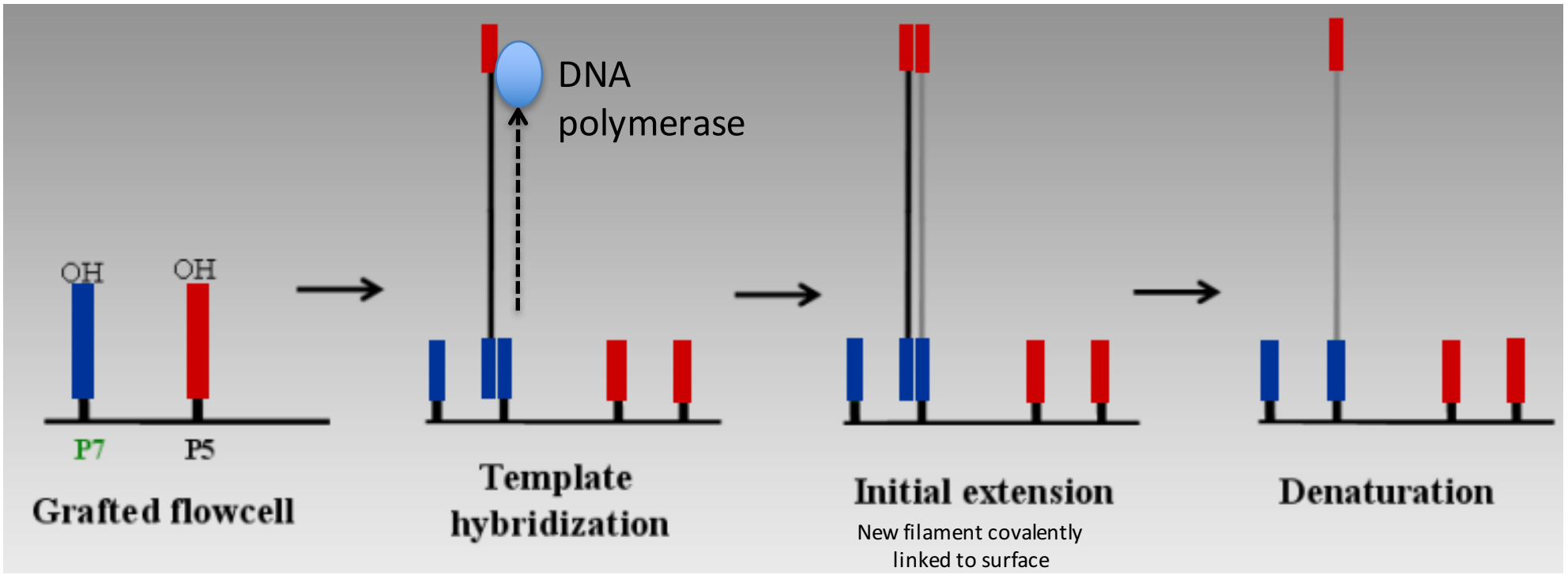
1 well, covered with
millions of 2 types of oligos

1 well in a flow-cell with
billions of wells

- ***complementary*** primers are ligated to the surface
- pairing with ChiP ed ssDNA at random position in the well of the flow cell

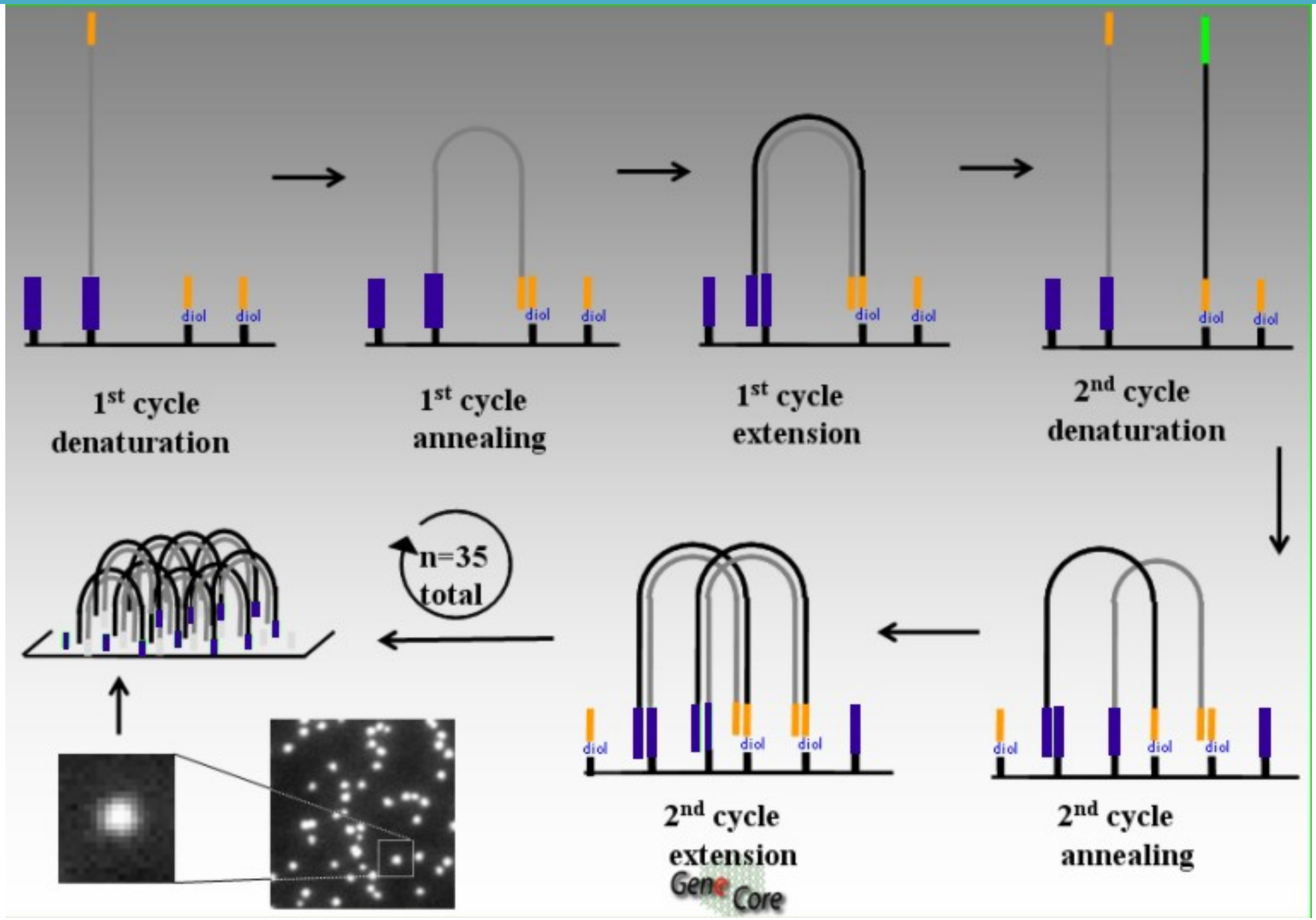
CLUSTER AMPLIFICATION:

Bridge amplification: takes place on surface of beads (each bead is mounted with 2 species of oligos; each oligo can hybridize to a DNA library fragment): initiation

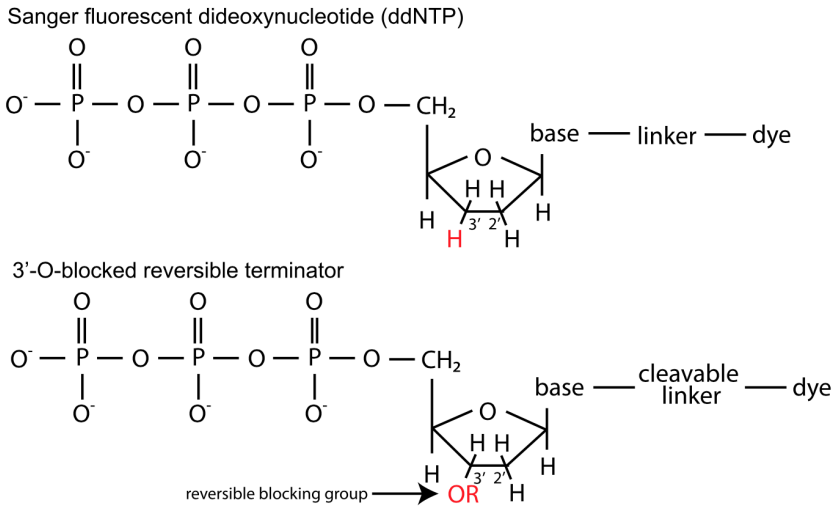


On the surface: complementary oligos

CLUSTER AMPLIFICATION:



REVERSIBLE CHAIN TERMINATORS:



Instead of promoting irreversible primer extension like the Sanger dye terminator method, the reversible chain terminators method uses a cydic method that consists of nucleotide incorporation, fluorescence imaging and cleavage.
The figure shows a modified nucleotide with a **cleavable dye** and **reversible blocking group. Once the blocking group is removed, a 3'OH is formed and a new nucleotide may come in.**

NOTE: no classic dNTPs are used for sequencing!!!!

Procedure

The steps for such a process can be outlined as follows:

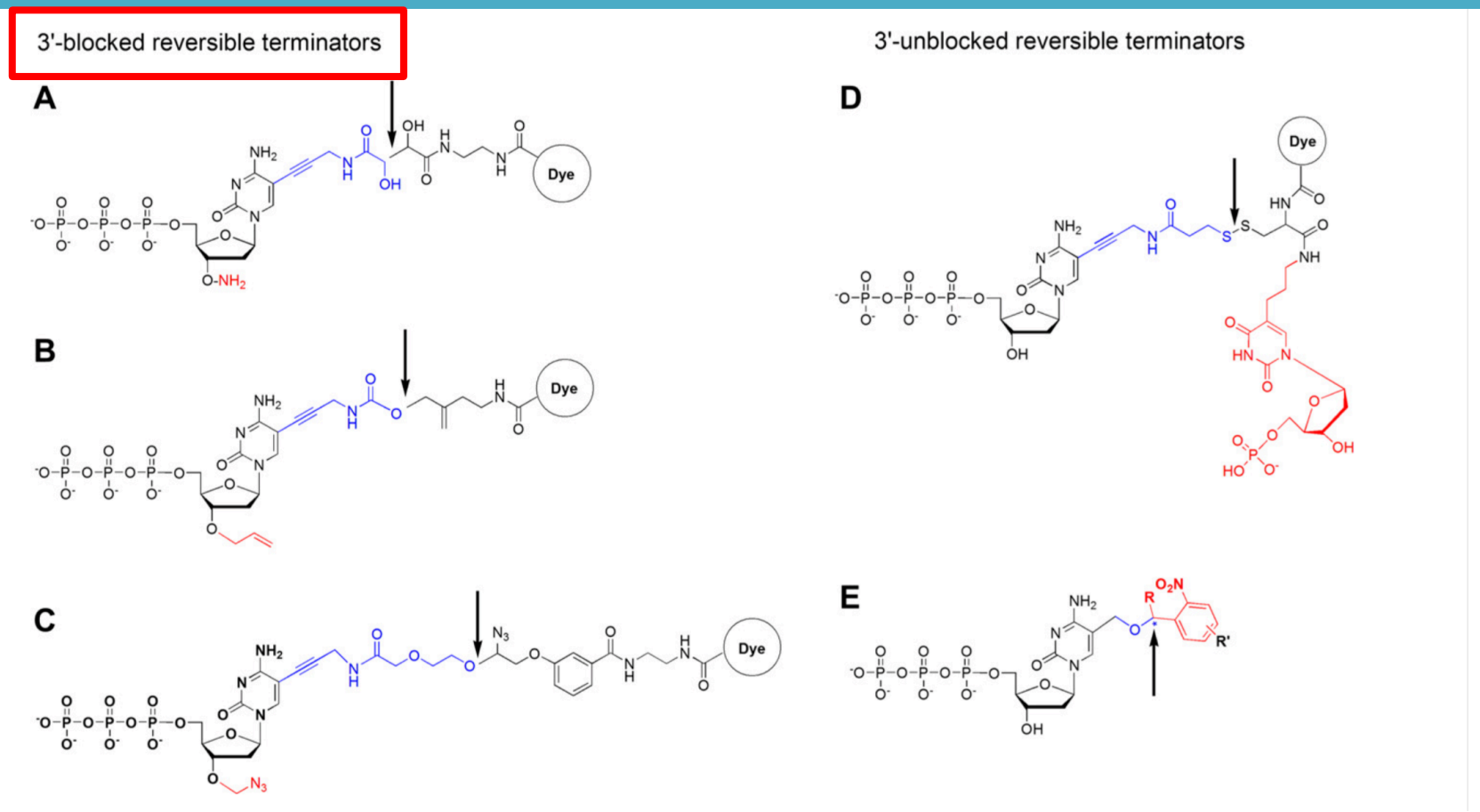
1. Have four dNTP's, each with a different fluorescent marking. These markings should not interfere with base pairing or phosphodiester bond formation.
2. Each dNTP should terminate DNA elongation temporarily with a blocking group on the 3' carbon of the sugar moiety.
3. Upon each cycle, have just one dNTP bind to the elongating strand and emit a fluorescent dye color.
4. Depending on the color emitted, record the particular nucleotide.
5. Cleave the blocking group and fluorescent dye with a palladium-catalyst.
6. Restore a 3' hydroxyl so that the growing strand can now elongate.
7. Repeat from step 1.

Cons

There are some limitations to this method which include:

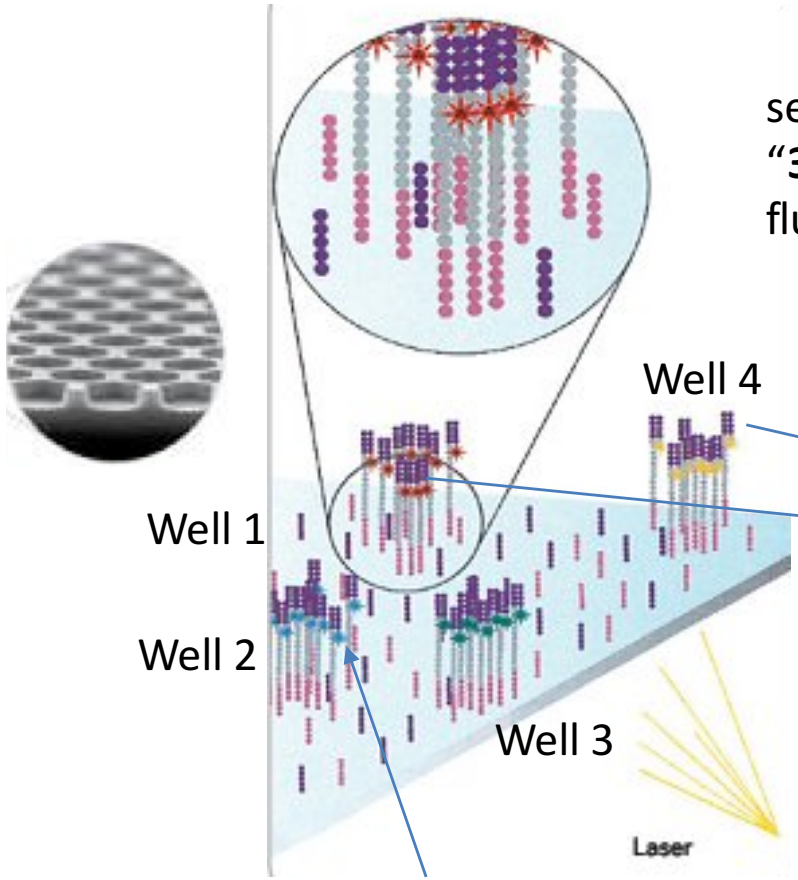
- Incomplete cleavage of blocking groups.
- Difficulties incorporating fluorescent nucleotides.

CLUSTER AMPLIFICATION:

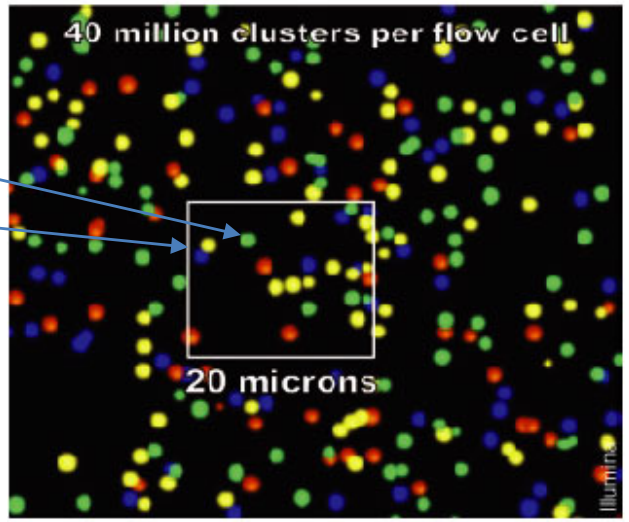


Three different 3’-blocked reversible terminators were shown on the left (A–C) and two 3’-unblocked reversible terminators were shown on the right (D–E).
The chemical structures in red denote the reversible terminating groups. Arrows indicate the site of cleavage separating the fluorescent groups from the nucleotide, and the chemical structures in blue denote the molecular scars that are attached to the base.

Illumina: massive parallel sequencing:



sequencing by synthesis with
"**3' blocked reversible terminator**" + cleavable fluorescently label (for each nucleotide)



1. **Start of synthesis using primer = incorporation of fluorescent 3'blocked reversible terminator: synthesis blocked**
2. **Scanning of fluorescent signals of all wells of flow-cell with laser (image)**
3. **Dye cleavage + elimination of reversible blocking group**
4. **wash step**
1. **Repeat steps 1-4 ca. 150x**

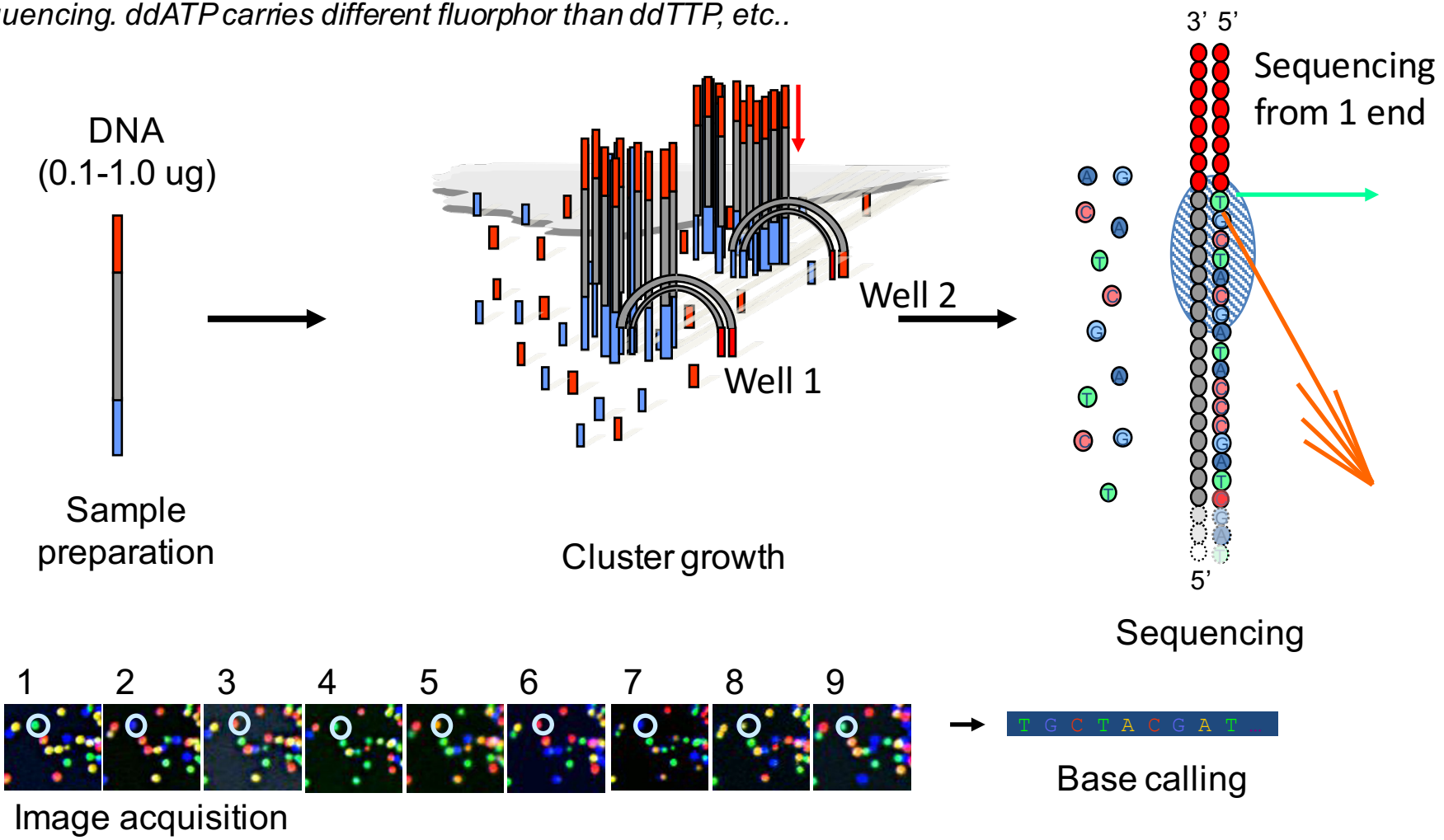
READ LENGTH: ca: 150nt from each primer (2x150nt = 300nt)

illumina.com

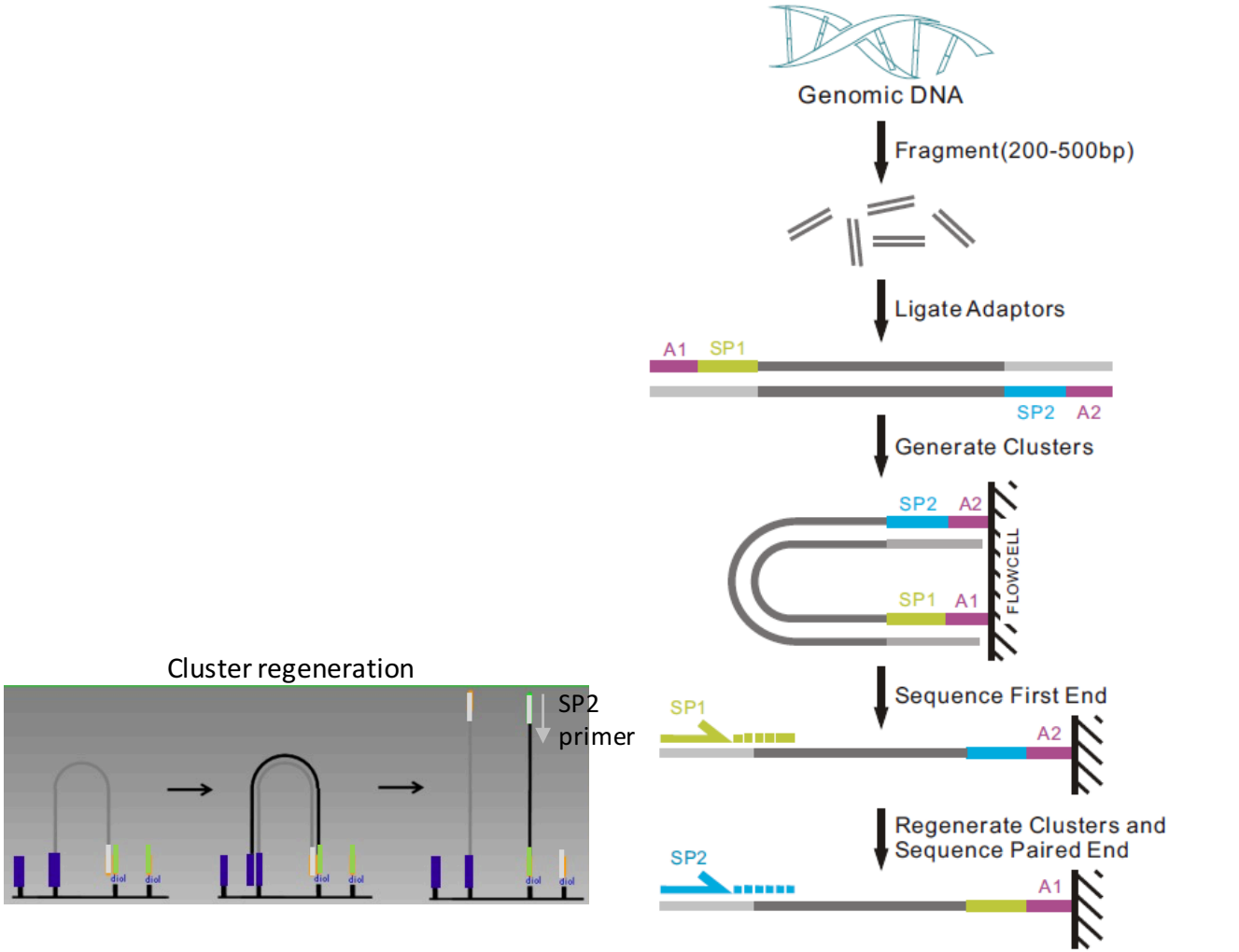
Illumina Sequencing Technology

Robust Reversible Terminator Chemistry Foundation

In each round of sequencing a fluorescently labelled ddNTP will be used for sequencing. ddATP carries different fluorphor than ddTTP, etc..



Illumina: paired end sequencing increases information content



1° strand sequencing by SP1

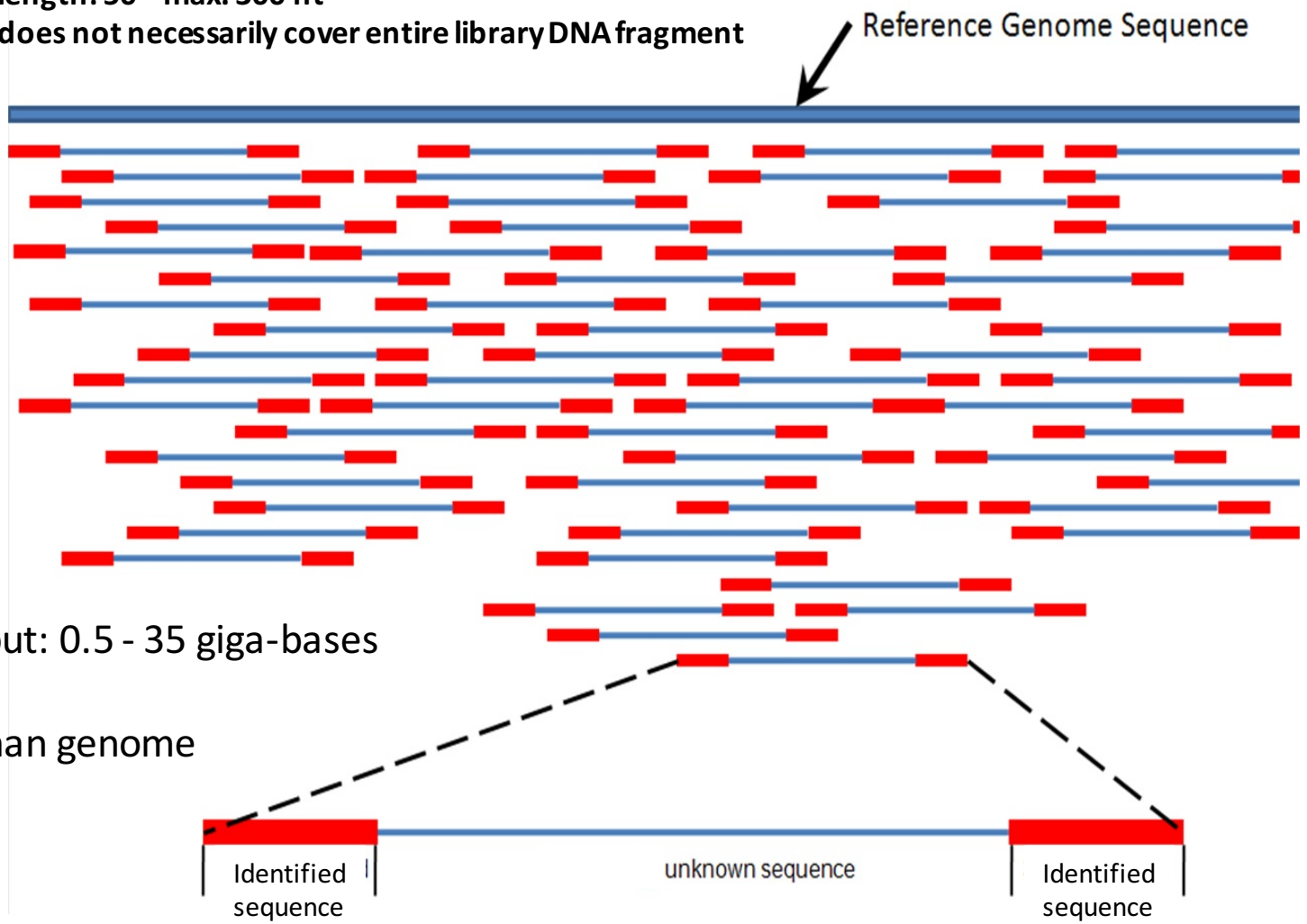
2° strand sequencing by SP2

Figure 1-2-1 Pipeline of paired-end sequencing (www.illumina.com)

https://www.youtube.com/watch?v=9YxExTSwgPM

Data analysis: obtained sequence reads are aligned along genomic DNA sequence → high number of reads necessary to obtain full sequence coverage

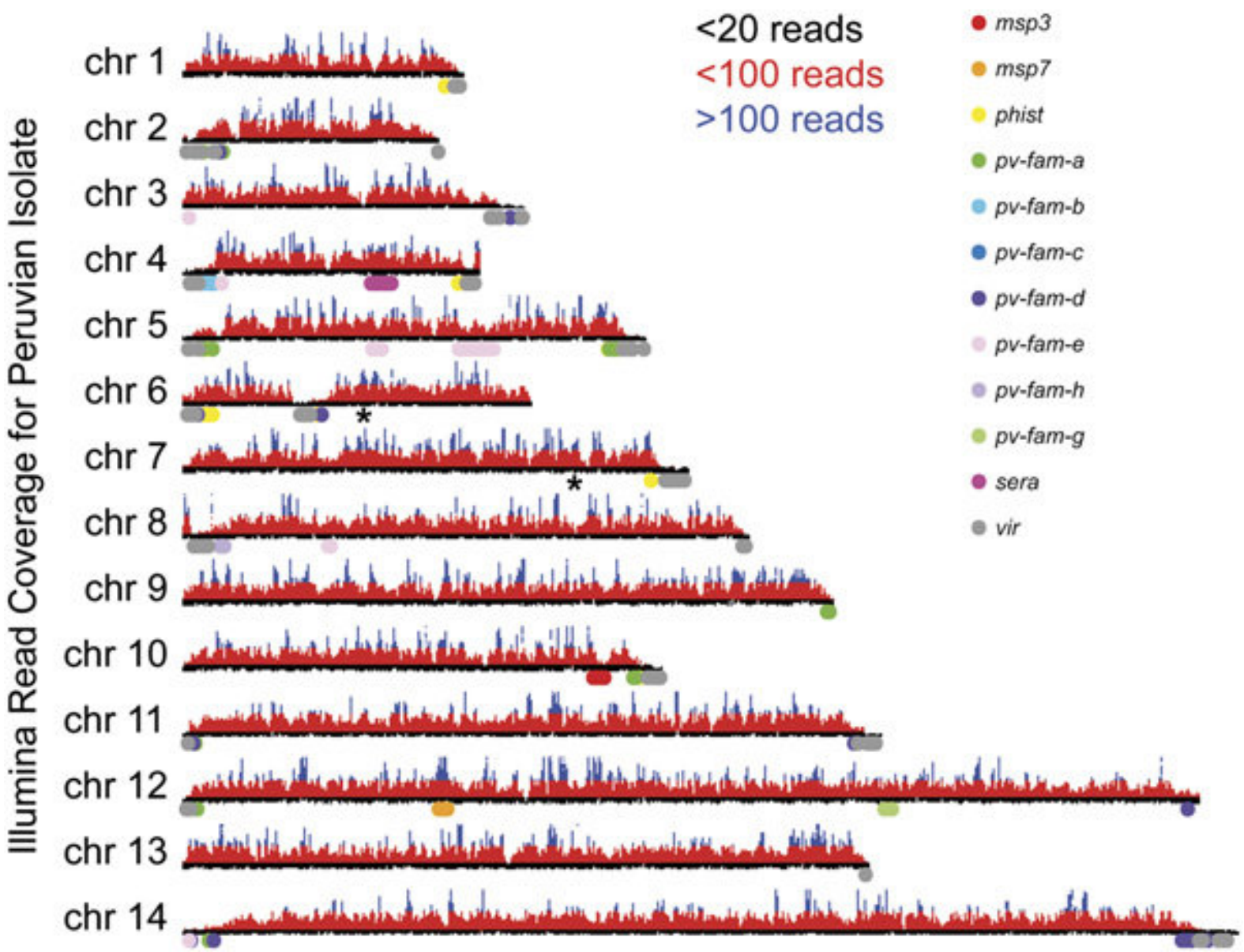
Read length: 50 – max. 300 nt
Read does not necessarily cover entire library DNA fragment



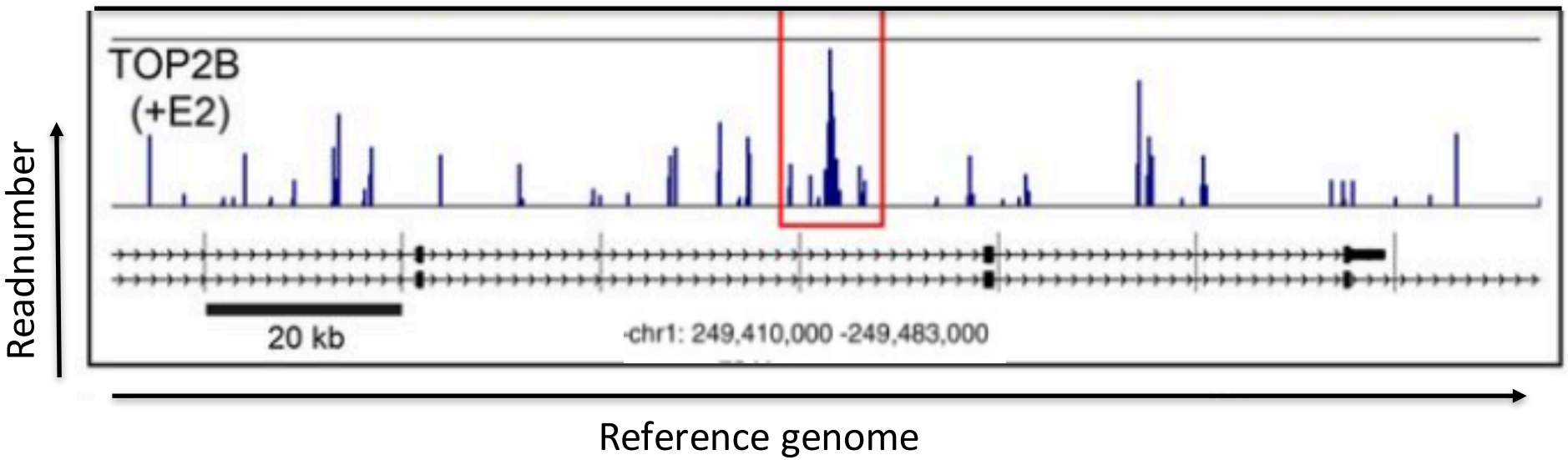
Max. output: 0.5 - 35 giga-bases
$=3.5*10^{10}$
= 10x human genome

Sequence derived from one amplified cluster

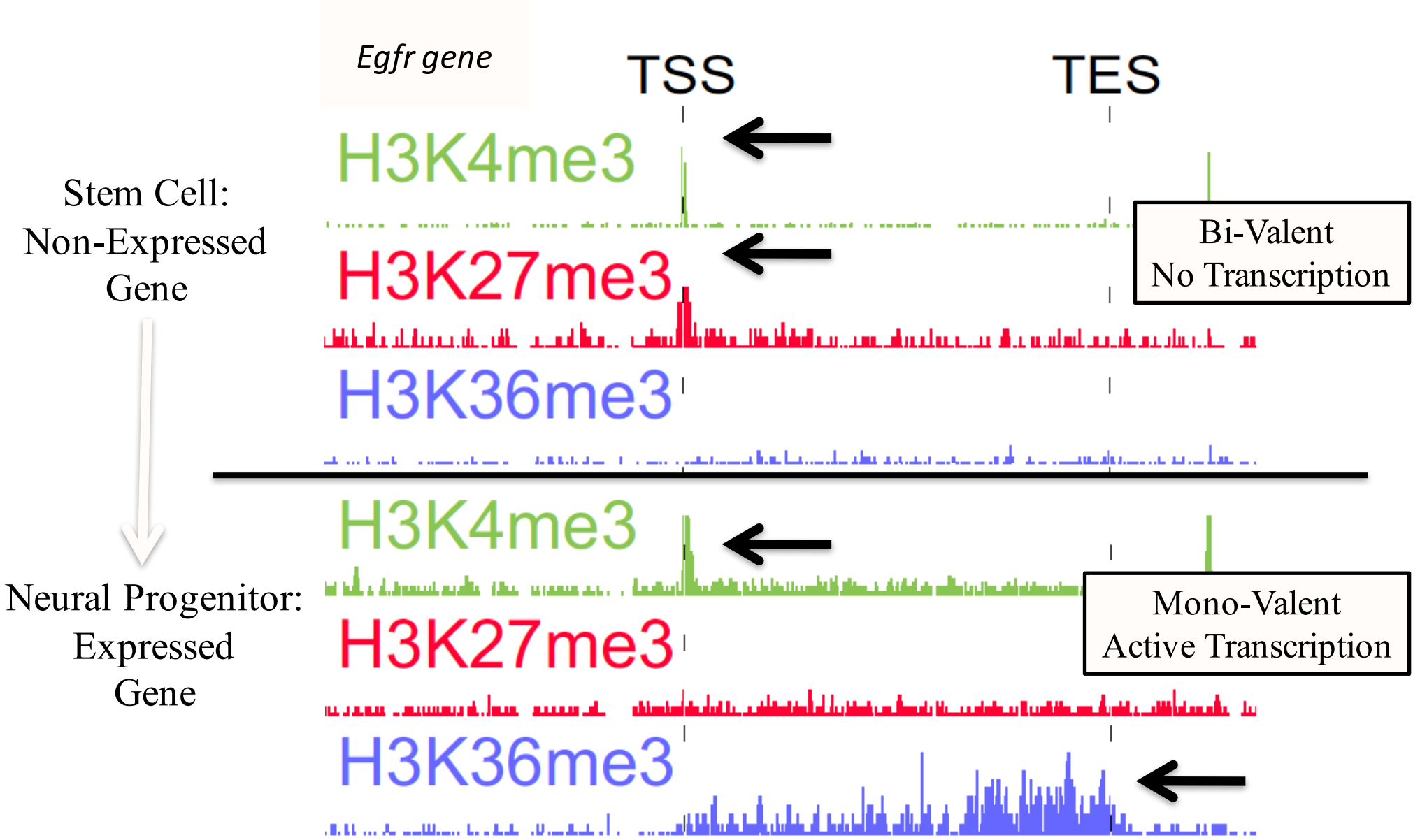
PILE – UP ALIGNEMENT ACROSS THE REFERENCE GENOME



Data analysis: obtained sequence reads are aligned along genomic DNA sequence → high number of reads necessary to obtain full sequence coverage

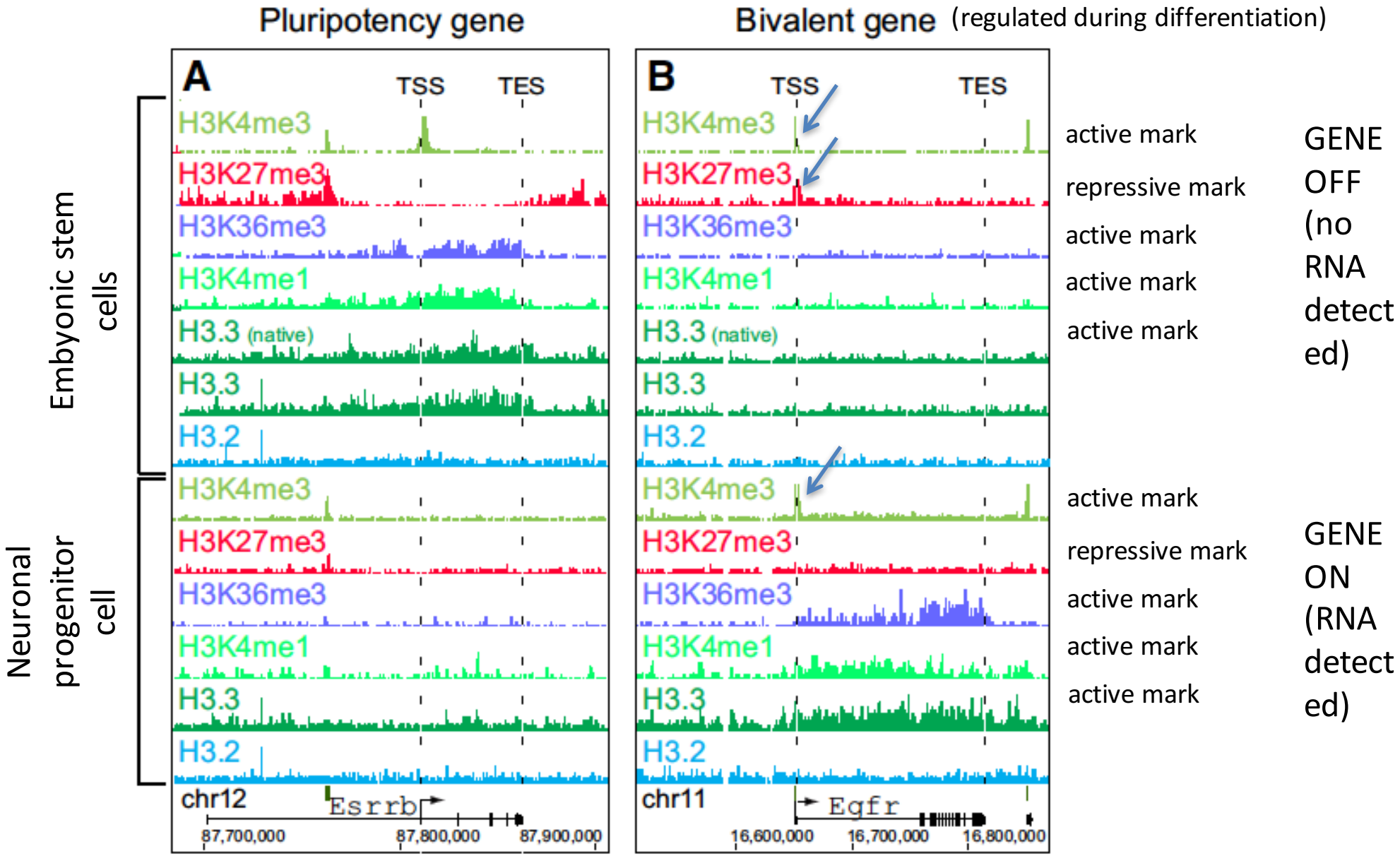


BIOINFORMATICS ANALYSIS:
Mapping ChIP seq reads agins the human genomic sequence



Goldberg et al., Cell, 140: 678-691. 2010

Mapping the epigenetic landscape enables to define "key rules" to define the epigenetic code of active and silent genes



Goldberg et al., Cell, 140:678-691. 2010