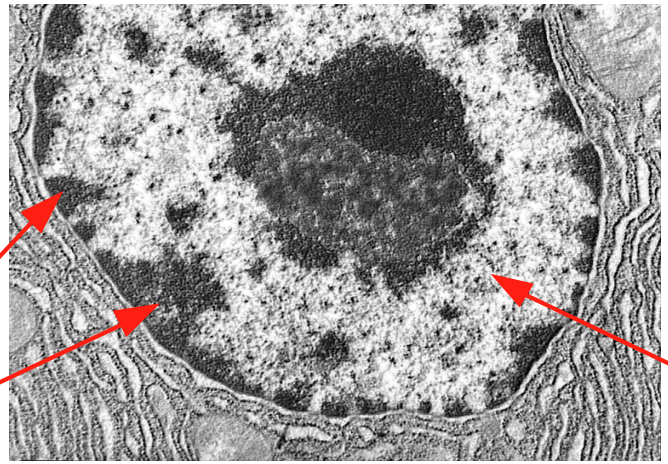
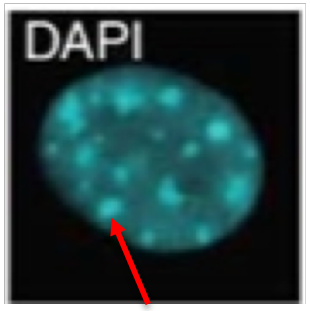
HOW TO STUDY EPIGENETIC MODIFICATIONS

-- 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:

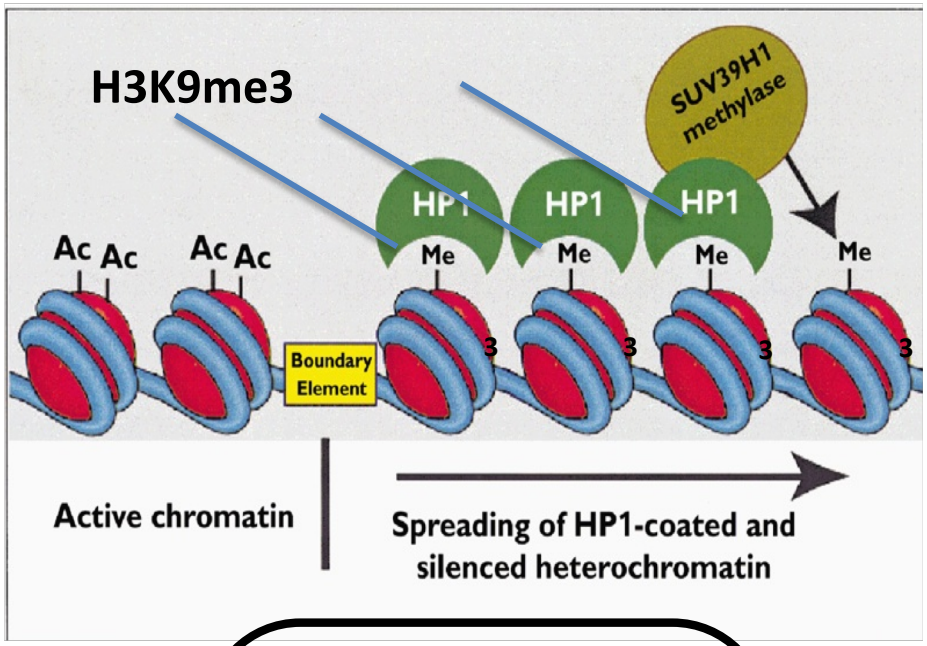
- constitute ~ 10% of nuclear DNA; telomeres, centromeres, and a considerable fraction of repetitive sequences
- highly compacted, replicates late in S phase, (transcriptionally inert)

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



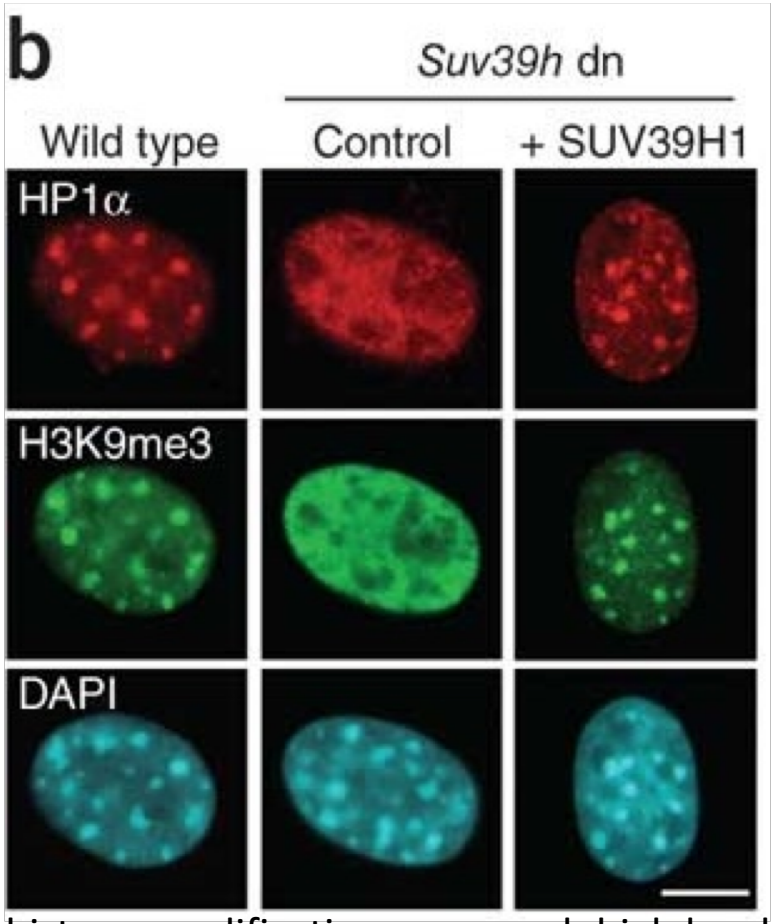
Suv39h1/2 tri-methylate Lysine 9 on histone H3

↓

tri-methylated Lysine 9 on histone H3 recruits HP1

↓

heterochromatin

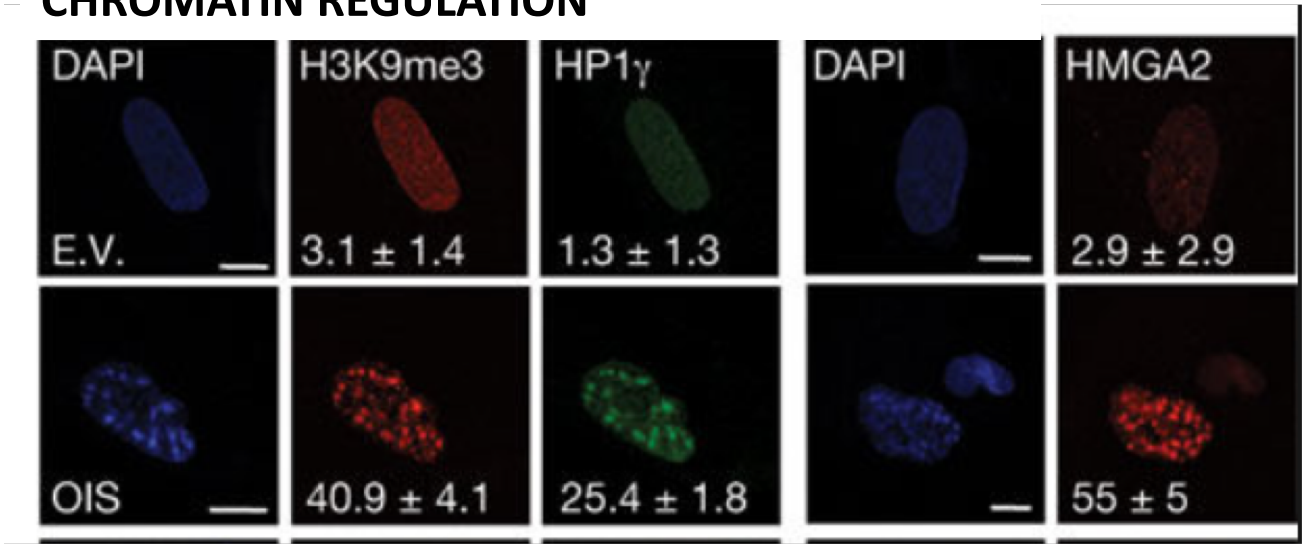


histone modifications can reach high levels in cells and can be visualized by immunofluorescence

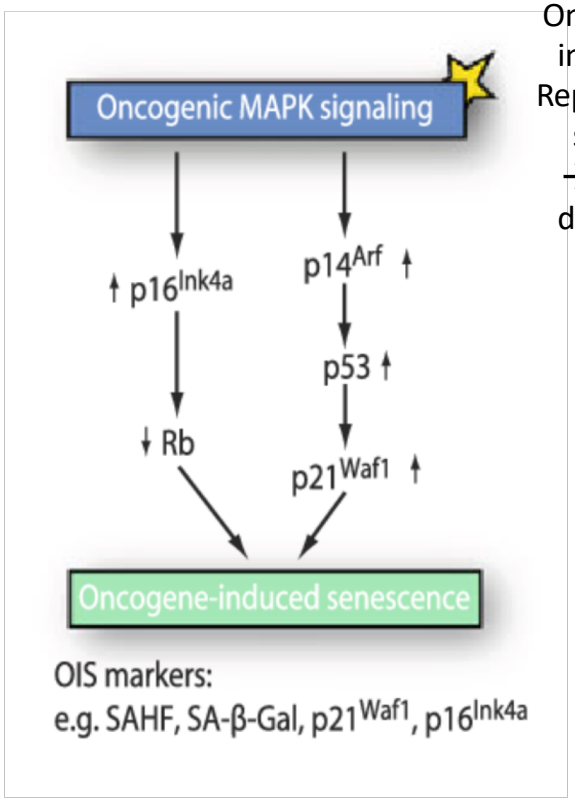
Post-translational histone modifications can recruit specialized proteins

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

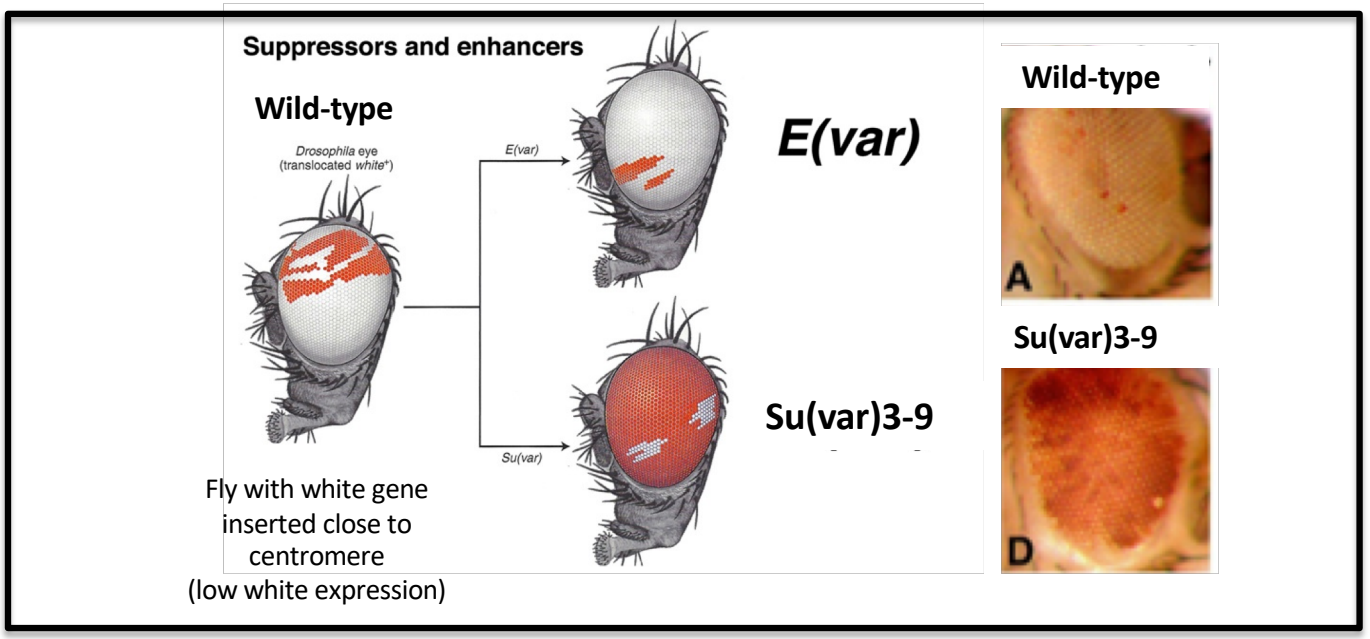
Lecture 3: Hallmark discovery and analysis of histone modifications

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

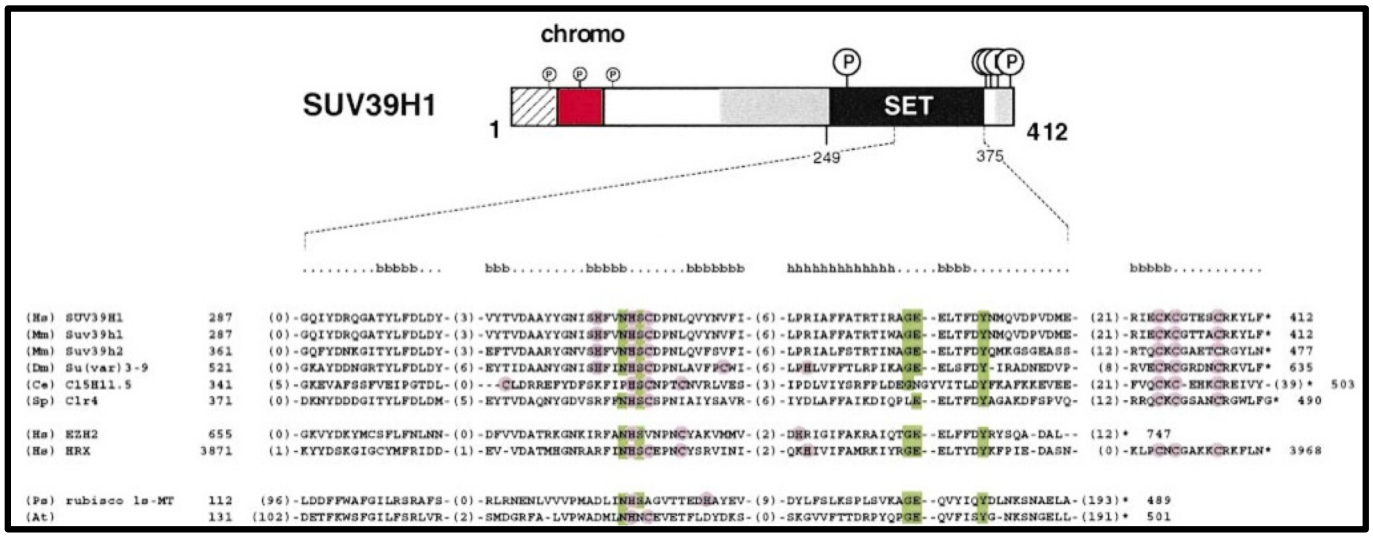
Identifying enzymatic for a "new" protein domain

Candidate gene:
Human
SUV39H1 and SUV39H2

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



Evidence 2
Conservation of SET domain
From yeast – plants – vertebrates
(IMPORTANT FUNCTION)



Evidence 3
Scientific report links SET domain to methylation (not histones)

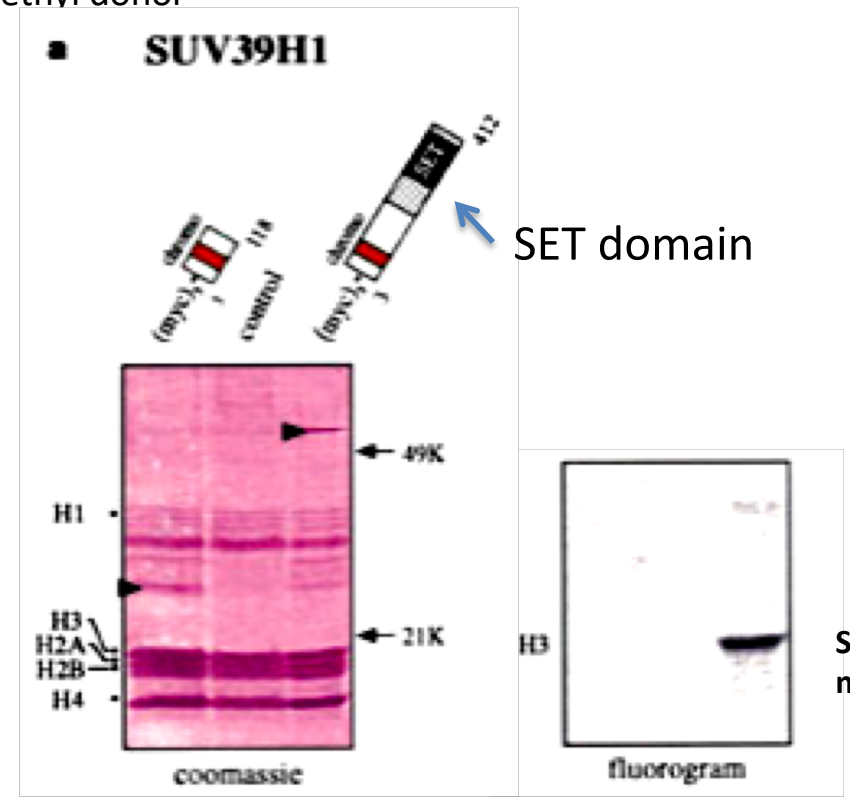
Identification of H3 Lys9 methyltransferase activity

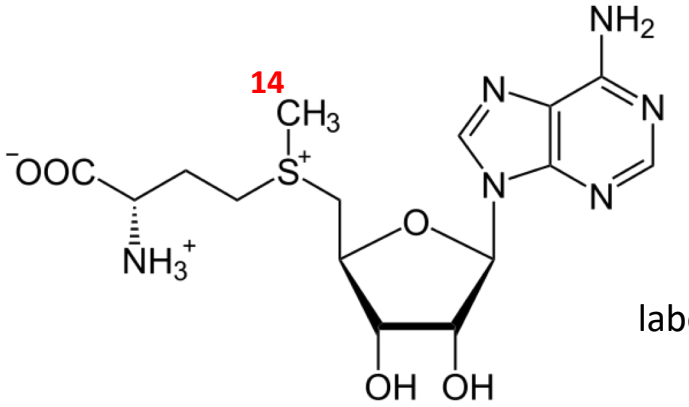
Experiment:

Overexpression of myc-tagged-SUV39H1 in Hela cells

Use an antibody to immunoprecipitate SUV39H1 → high concentration of SUV39H1

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





Alternative: labelling with H^3

SET – domain is required for histone methyl transferases activity

Note: ALSO APPLICABILE FOR HATs AND Acetyl CoA or other enzymes

- 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.

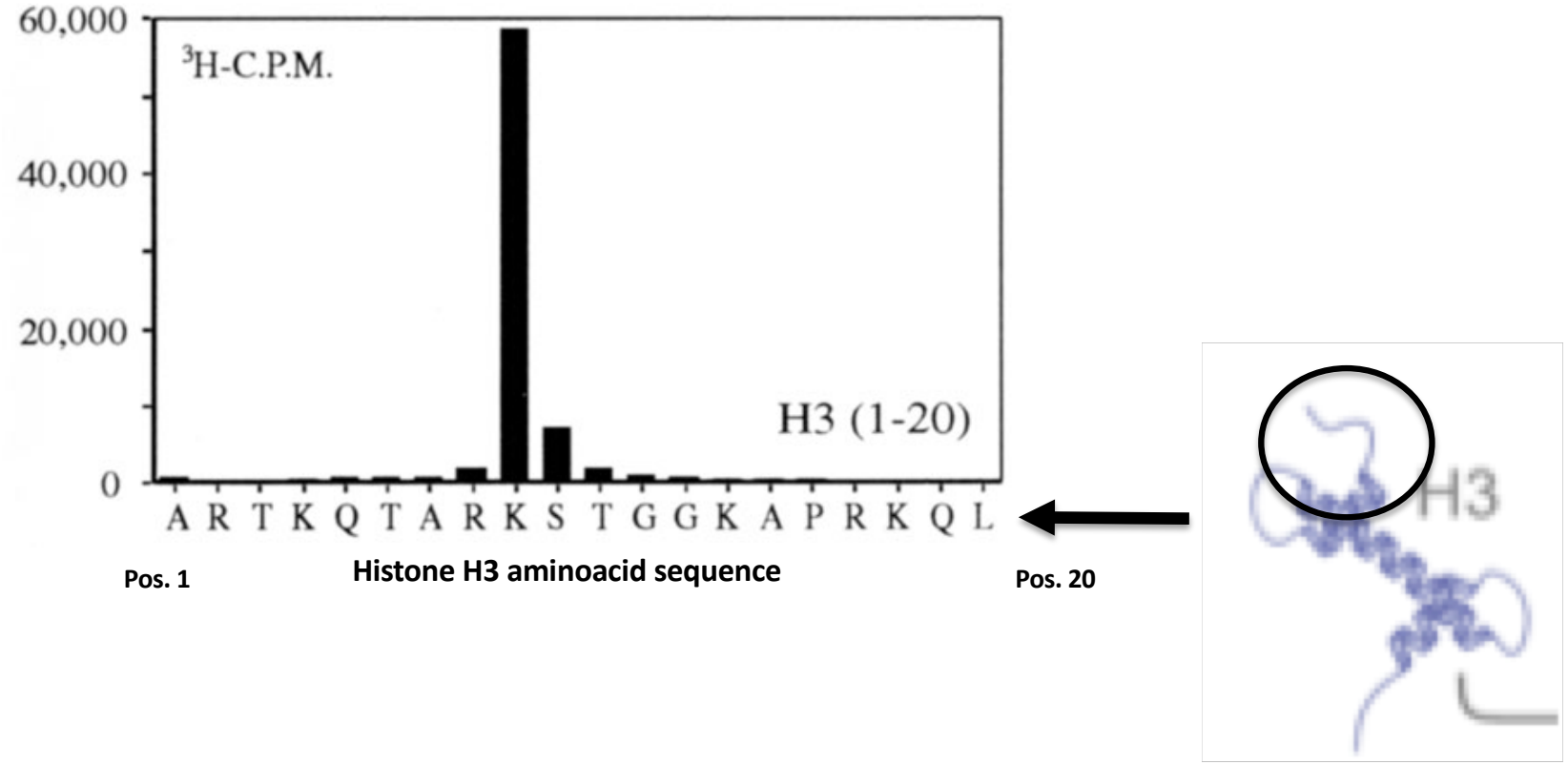
Identification of H3 Lys9 methyltransferase activity

Experiment:

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

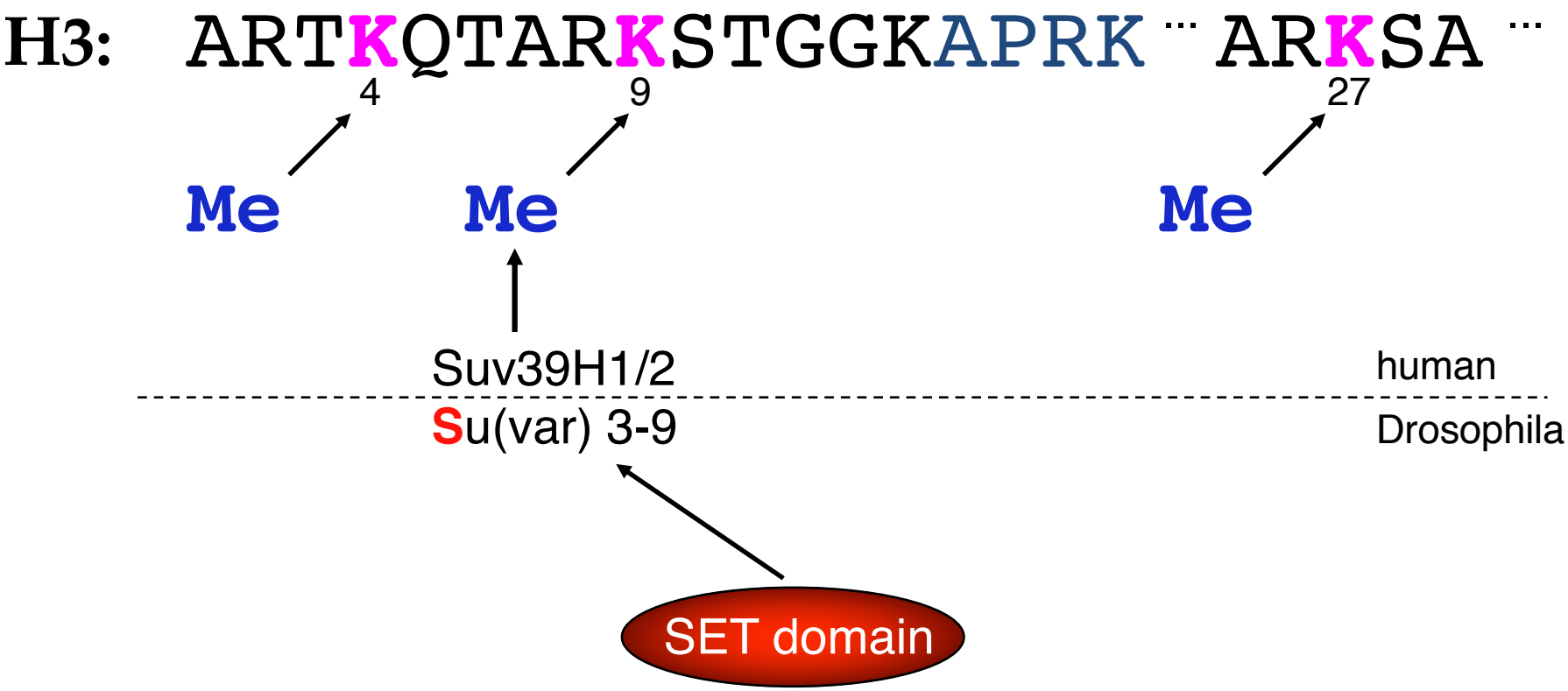
Chemically clip off amino acids one-by-one, identify aminoacid identify and quantify the amount of radioactivity

Suv39H1 methylates histone H3 at lysine 9



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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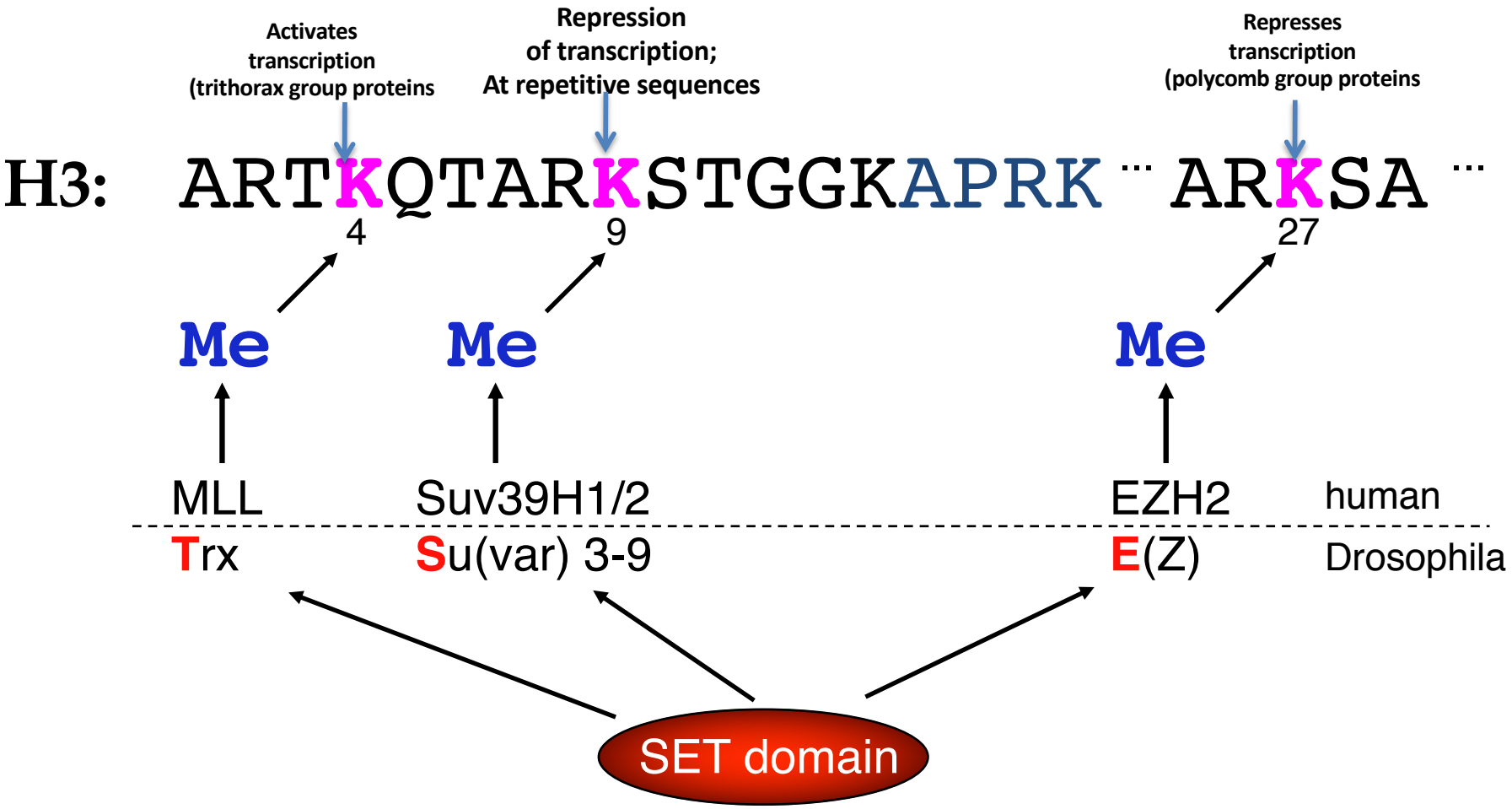
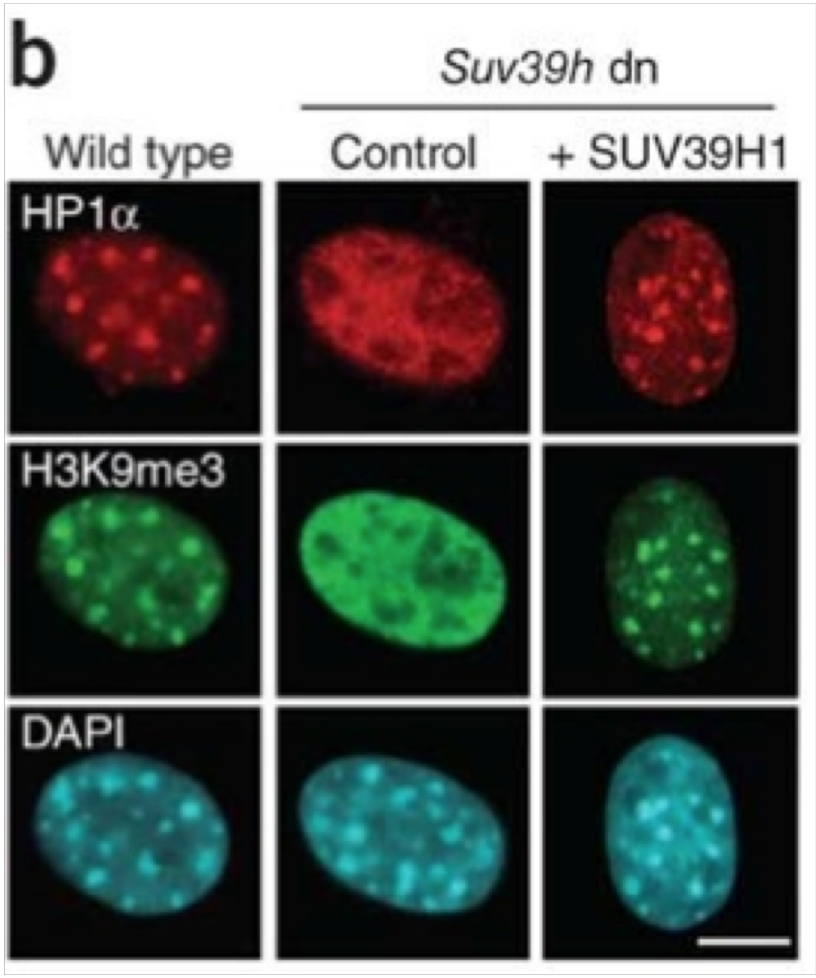


- Mutations of some histone methyltransferases → cancer

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?

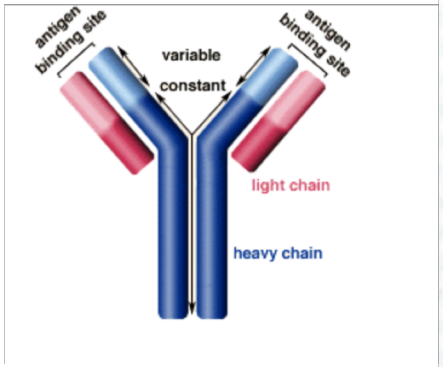
-Chromatin immunoprecipitation

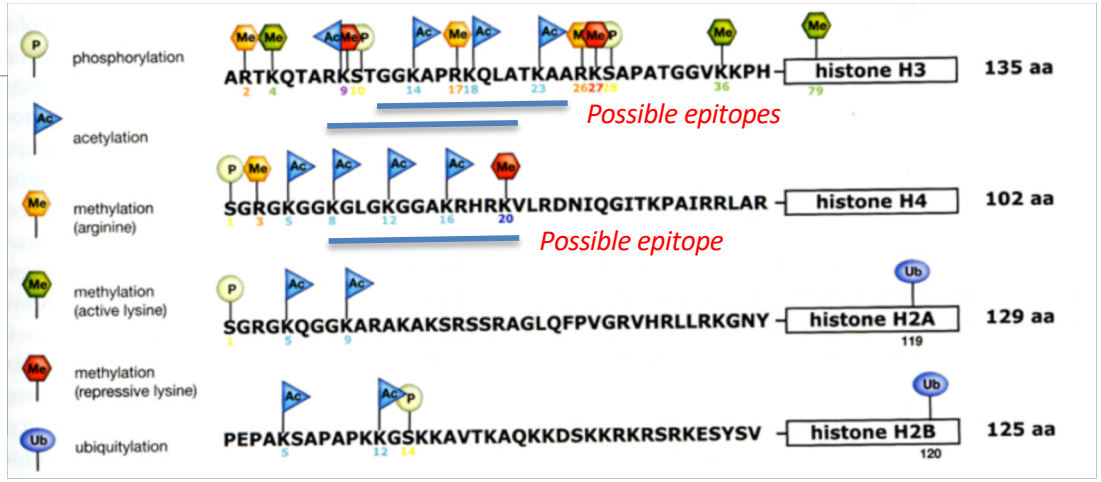
--> Detect histone modifications on single genomic site

--> Detect histone modifications on multiple sites or on the entire genome level

Lecture 3: Hallmark discovery and analysis of histone modifications

1. Generation of antibodies that specifically recognize modified histones

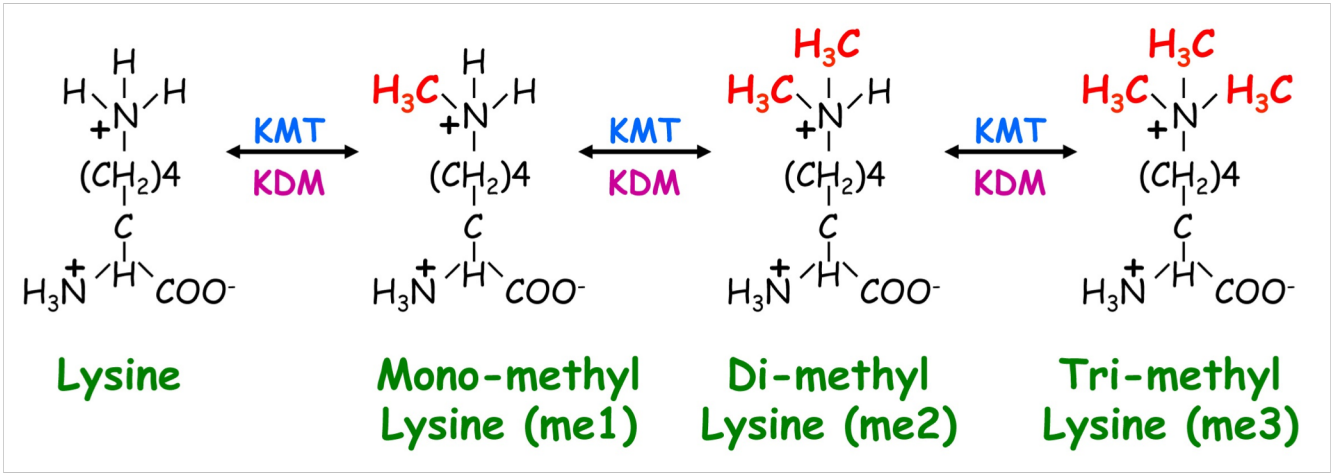




CAN WE GENERATE MODIIFCATION SPECIFIC ANTIBODIES??
→ Detect a short amino acid sequence (ca 15aa) with specific modification

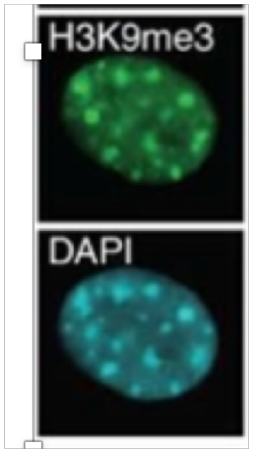
Problem 1: Lysines can be mono-, di- and tri-methyalted
An example: H3K9methylation by Suv39h1

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



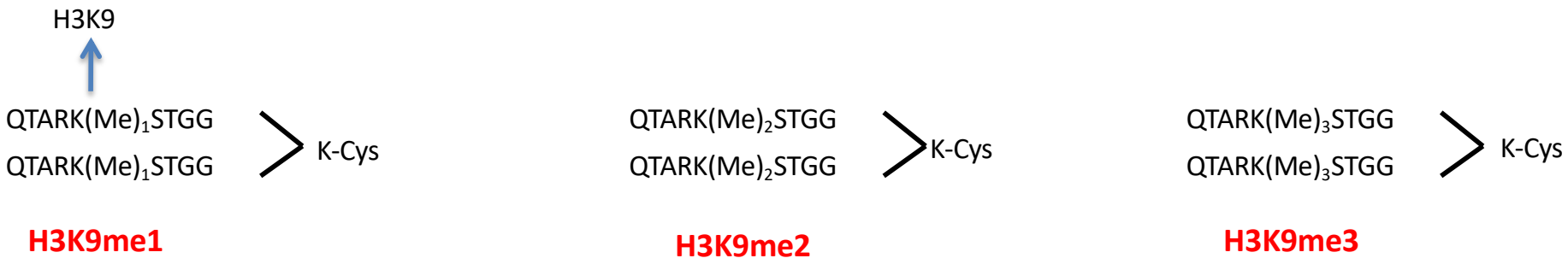
1. Generation of antibodies that specifically recognize modified histones

Problem 2: In vivo repressive histone modifications compact chromatin = nucleosomes are thought to be “packed”



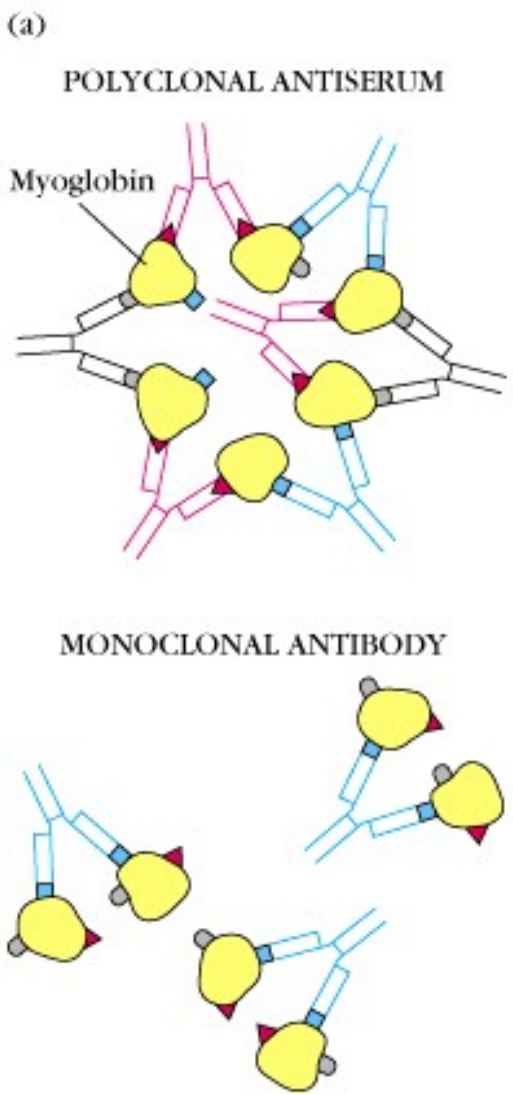
= particular epitope in living cells
→ Additional information for antibody specicity

SYNTHESIS OF BRANCHED PEPTIDES FOR ANTIBODY GENERATION



→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

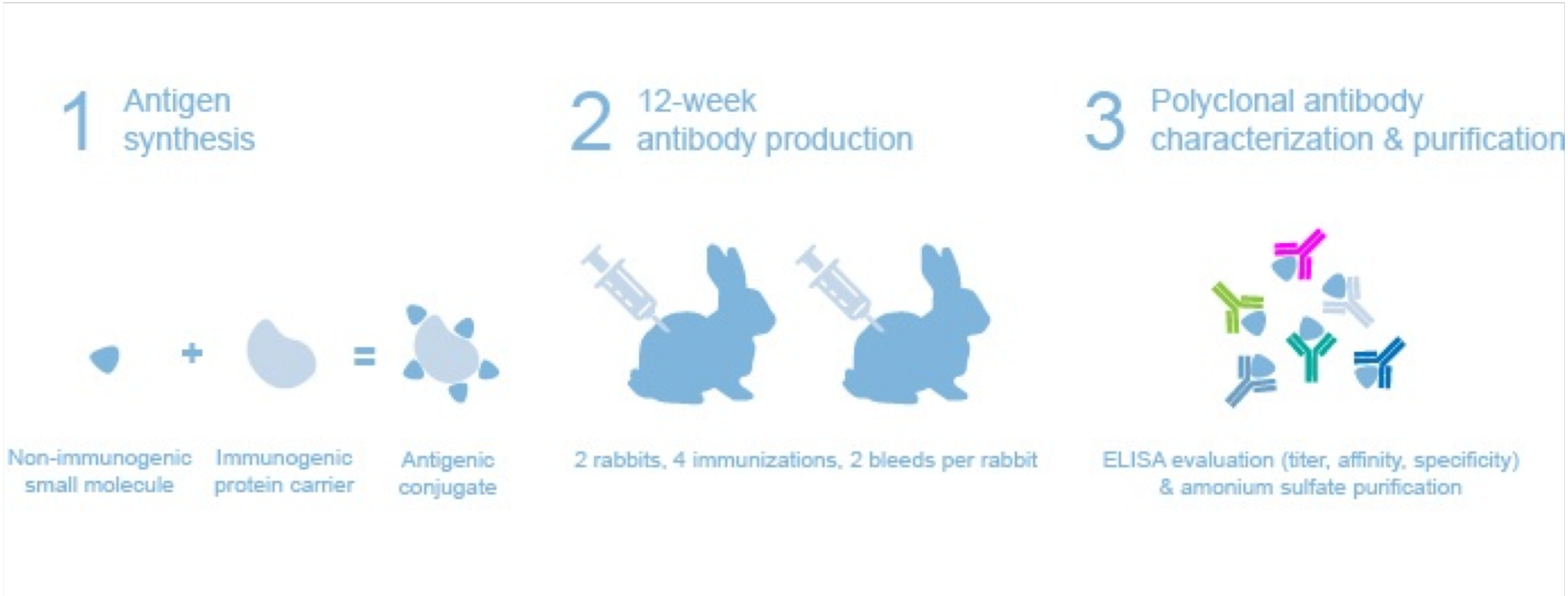
Animal 1

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

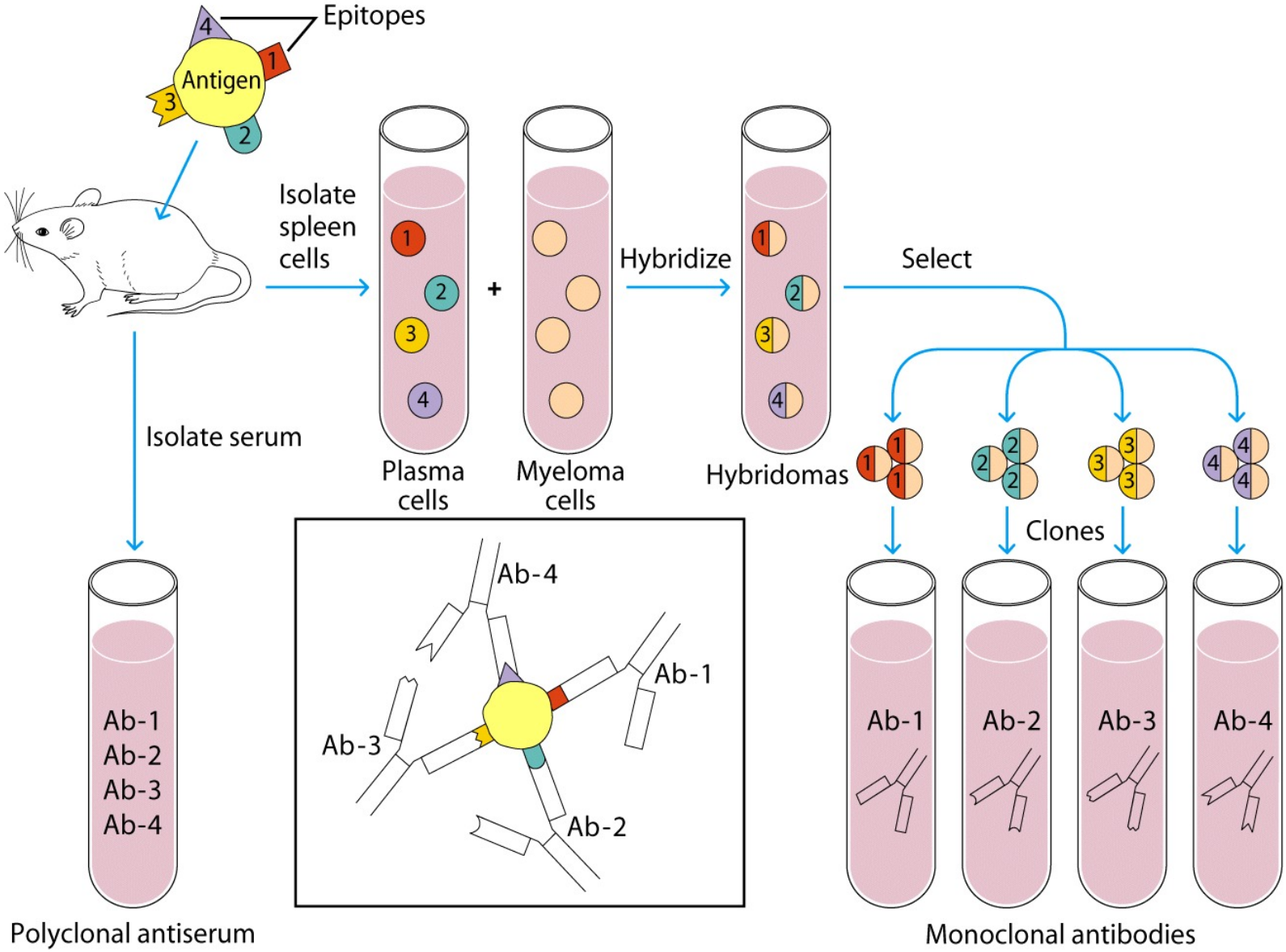
H3K9me3

Animal 2

control



Generation of monoclonal antibodies



Generation of monoclonal antibodies

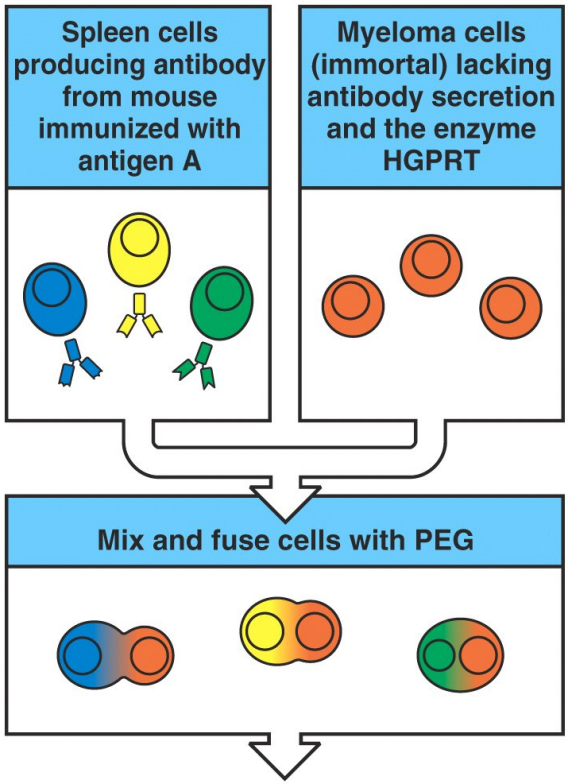


Figure A-14 part 1 of 2 Immunobiology, 6/e. (© Garland Science 2005)

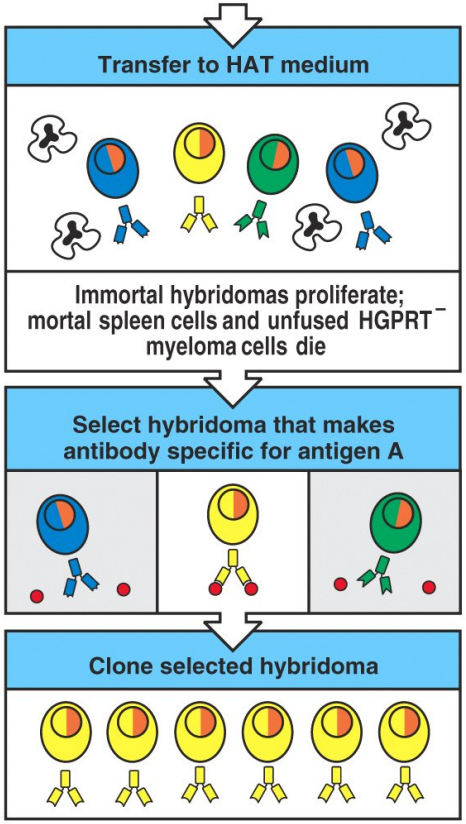
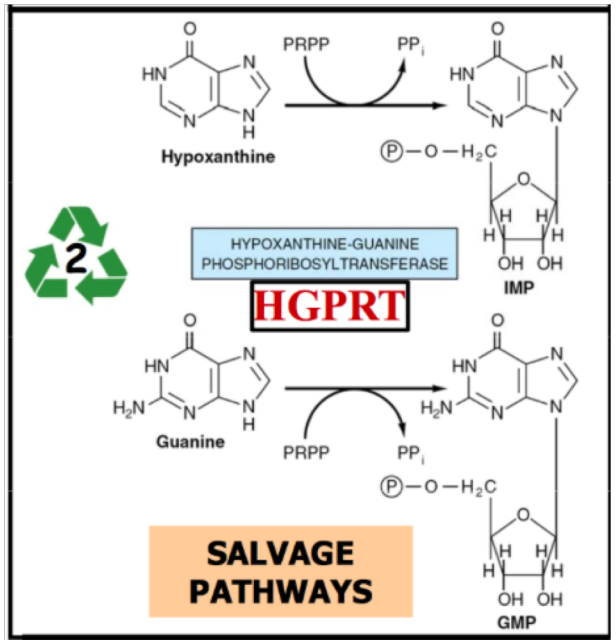


Figure A-14 part 2 of 2 Immunobiology, 6/e. (© Garland Science 2005)



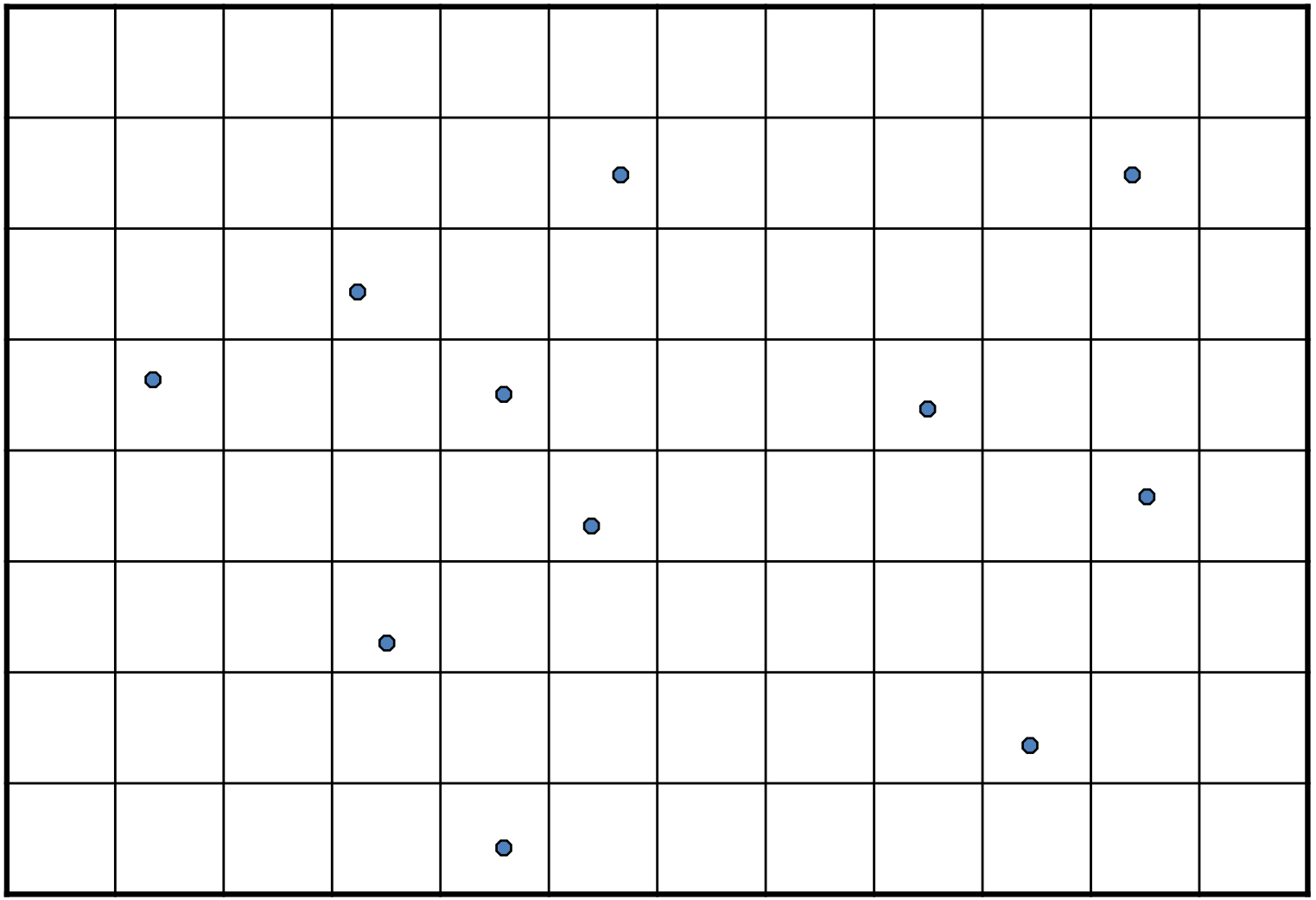
Most purines are recycled rather than degraded. HGPRT can recycle hypoxanthine and guanine to be used in DNA replication

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. **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 right enzymes, which means having functioning copies of the genes that encode them. HGPRT: inactivates aminopterin → 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
Plate at limiting dilution (<1 cell/well) in 96 well plates.
Allow clones to expand.
Expand positive well and test for production of antibody of desired specificity in culture supernatant



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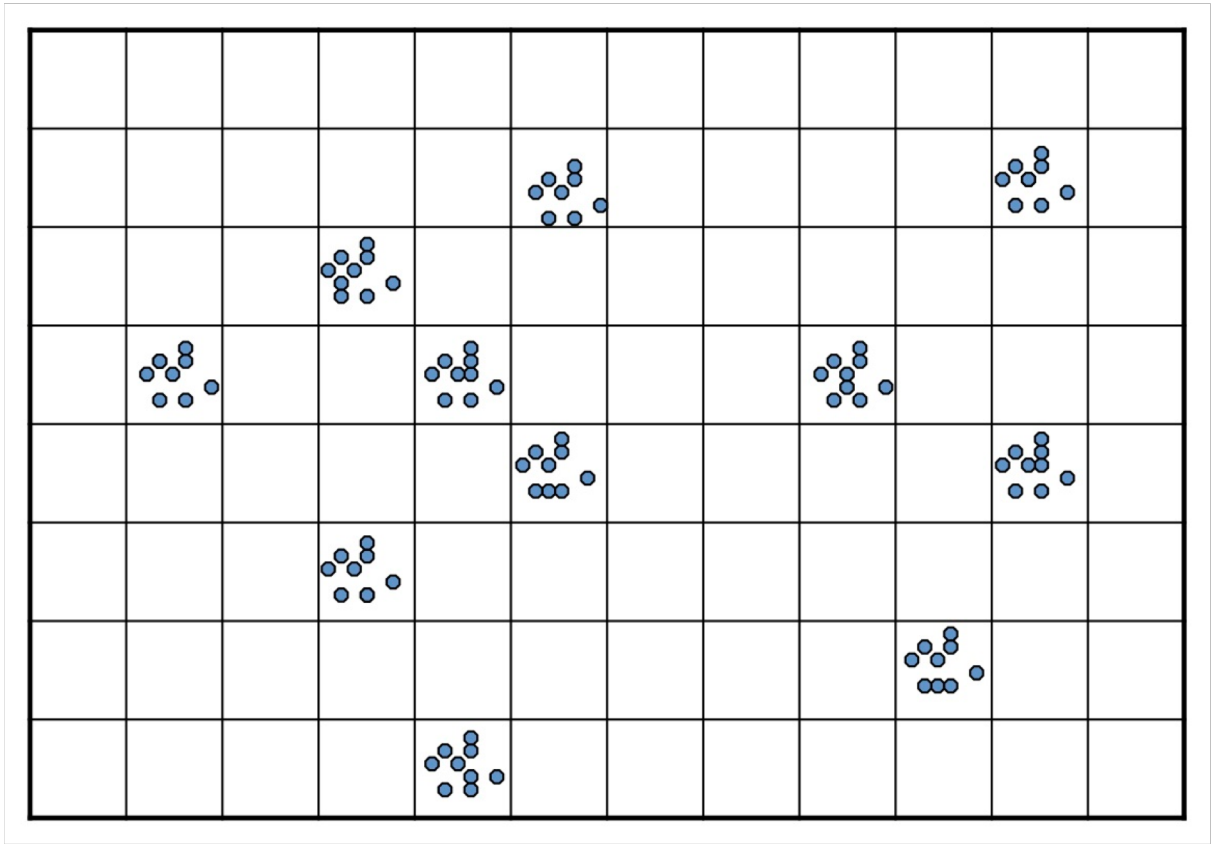
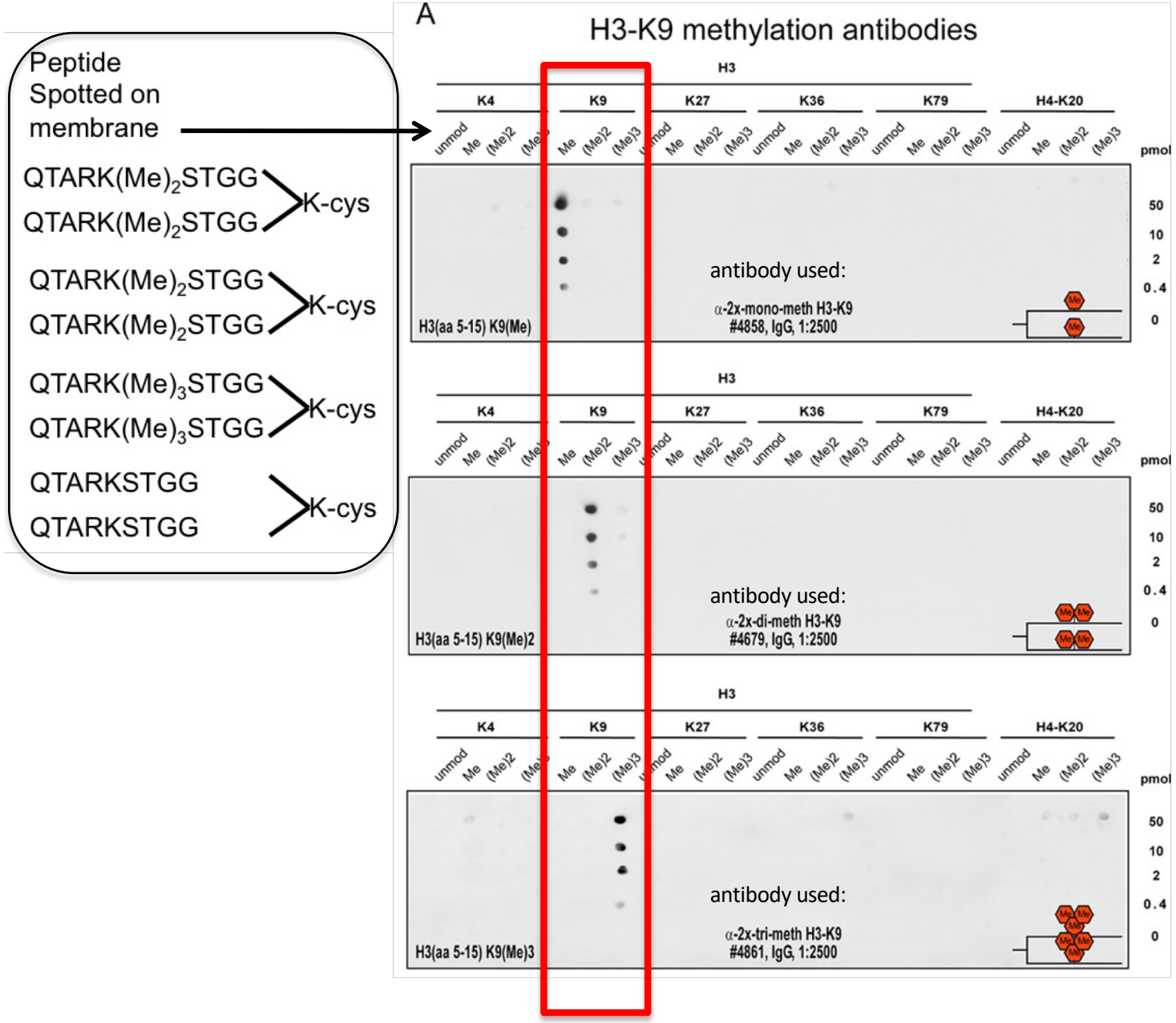


-Hybridoma cells secrete antibody into cell culture medium
-Purification of antibody
-Validation of antibody

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

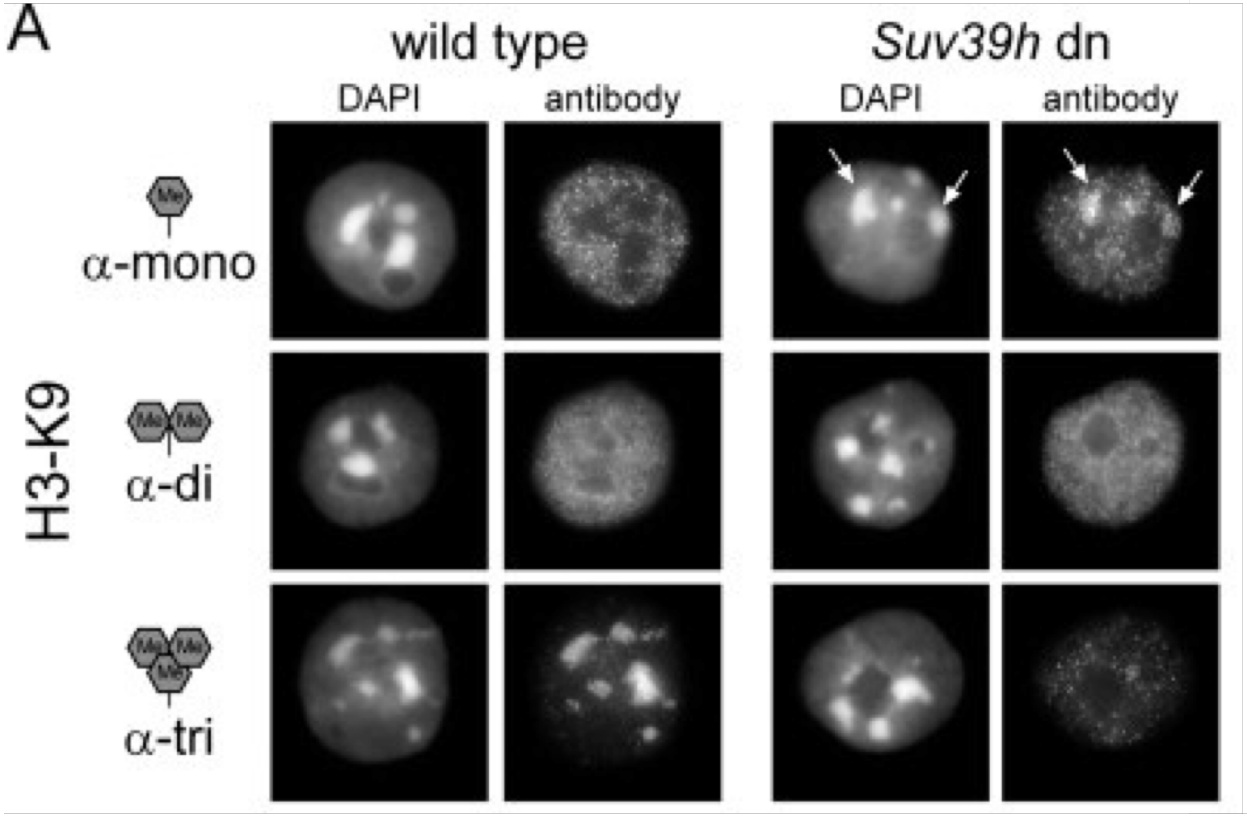
3. Incubate with secondary Antibody that is coupled With horseradish peroxidase

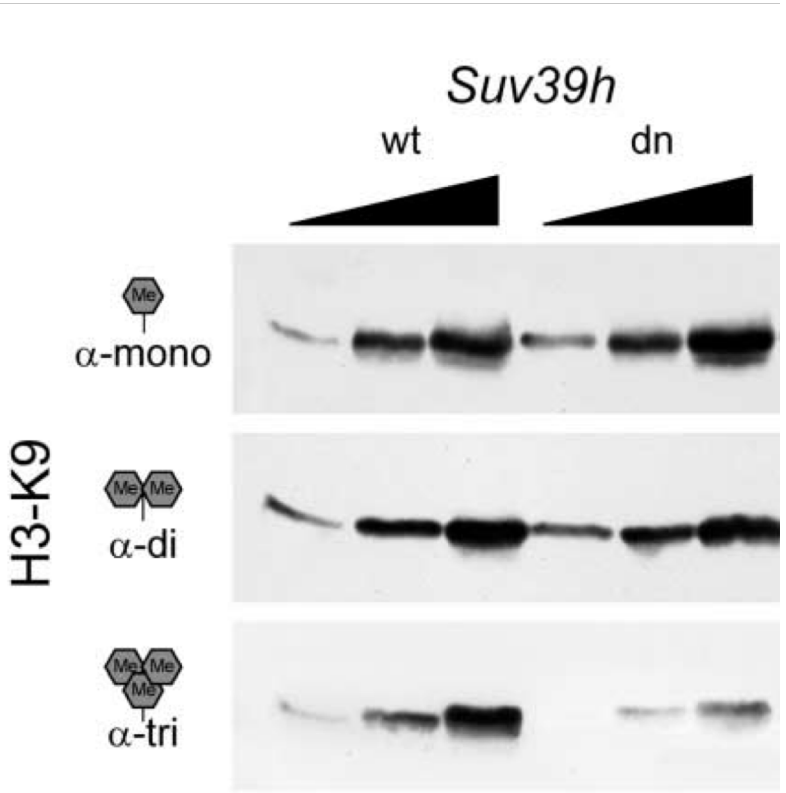
4. Add substrate for Horseradish peroxidase

5. Develop

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

Suv39h1/Suv39h2
Knock-out cells

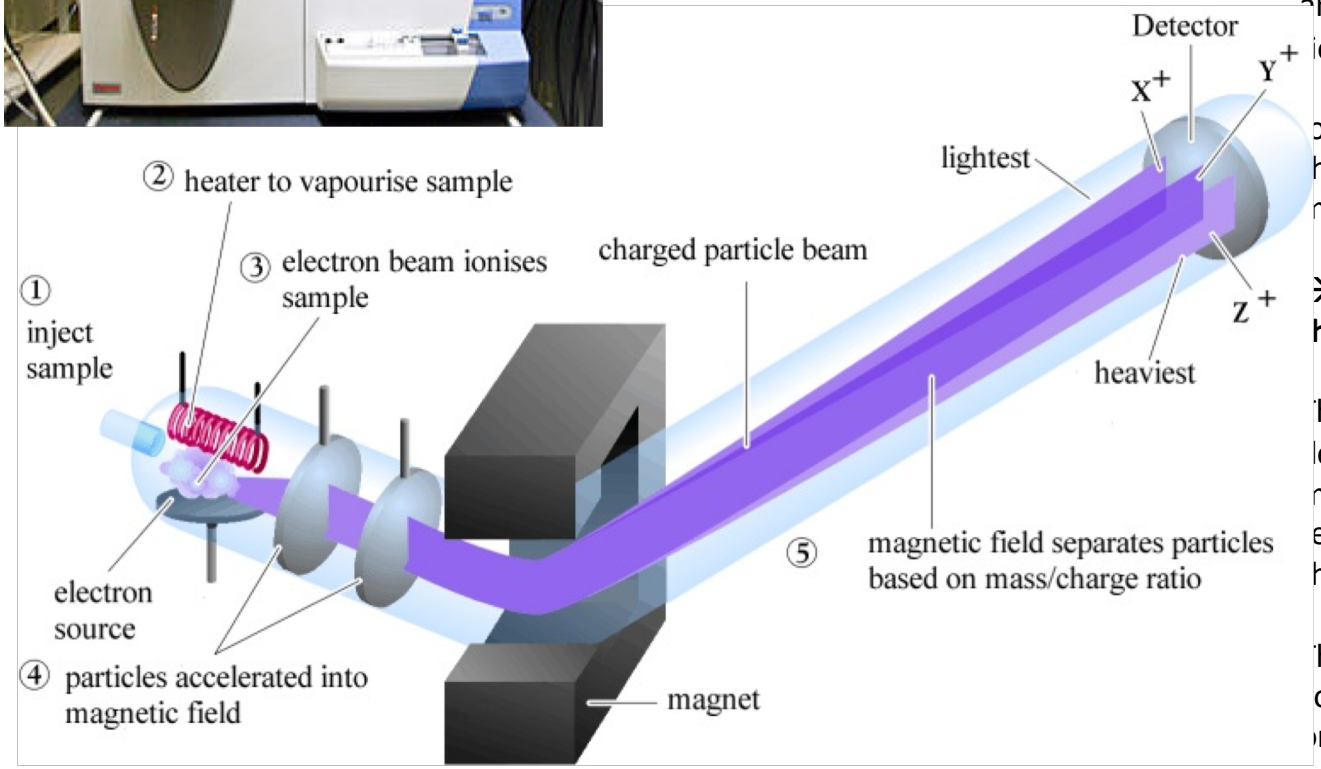




In Suv39h dn cells, H3K9me2 and H3K9me2 are still present.
Suv39h1 is a H3K9 specific HMTase that is required to establish the TRI-METHYLATION of H3K9 in mammalian cells

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

2. Studying histone modifications by mass spectroscopy



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 nd subjecting them to an electric or magnetic ield:

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

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

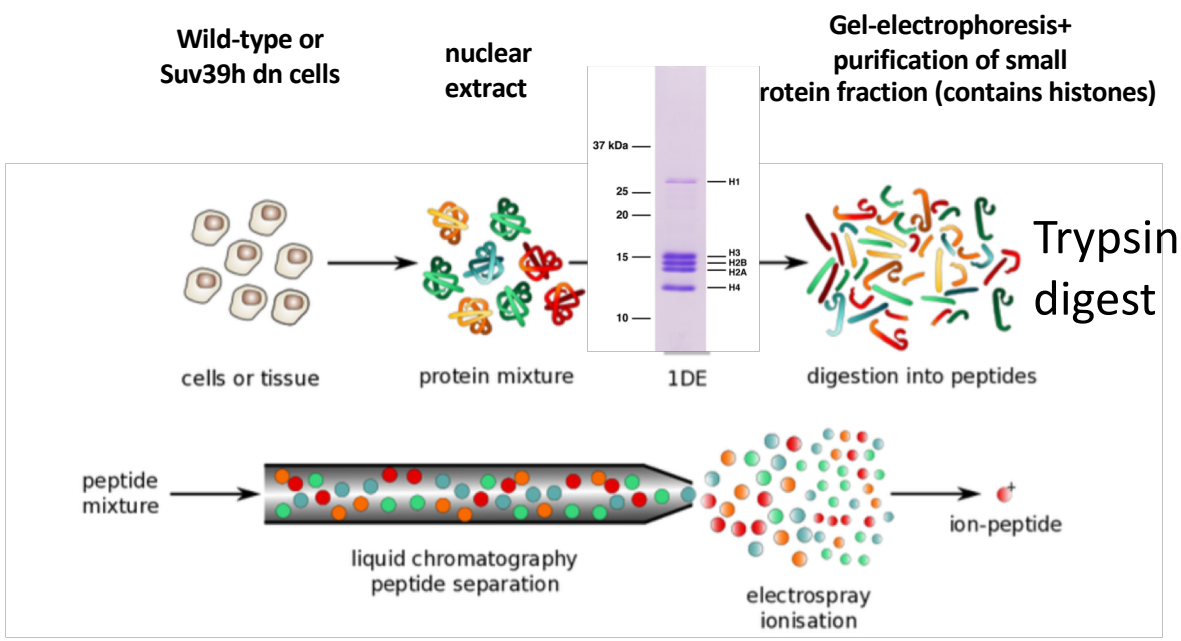
he ions are detected by a mechanism capable of etecting charged particles, such as an electron ultiplier. Results are displayed as spectra of the elative abundance of detected ions as a function of he mass-to-charge ratio.

he molecules in the sample can be identified by orrelating known masses to the identified masses r 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 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

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

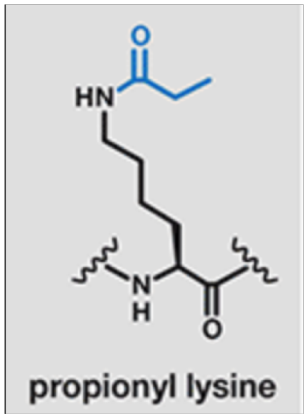
2. Studying histone modifications by mass spectroscopy

PROBLEM:

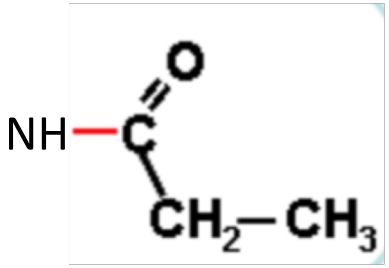
1.Trypsin cleavage after K is not complete because me2 or me3 on some lysines block block cutting by Trypsin

2. Many cleavages (me0, me1) → 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



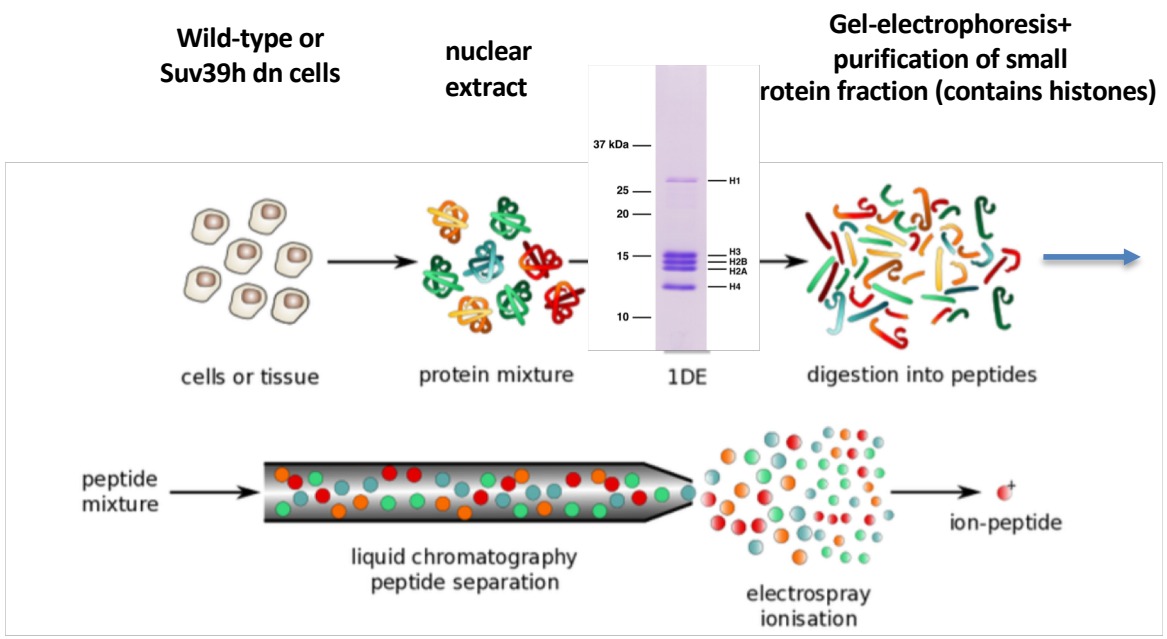
Proprionyl group



SOLUTION: In vitro Proprionylation of unmodified or mono-methylated lysine prevents trypsin cleavage AND creates a mass:charge ratio that allows to differnetiate between fragments carrying me1, me2 or me3 marks

Now Trypsin can only cut after Arginine. This allows a uniform cleavage of histone tails

2. Studying histone modifications by mass spectroscopy



Propionylation → ***Trypsin digest***