

# Epigenetics in toxicology: Principles, technology, data analyses, and challenges

Maastricht University

Simone van Breda

# Content

## **1. Background on epigenetics**

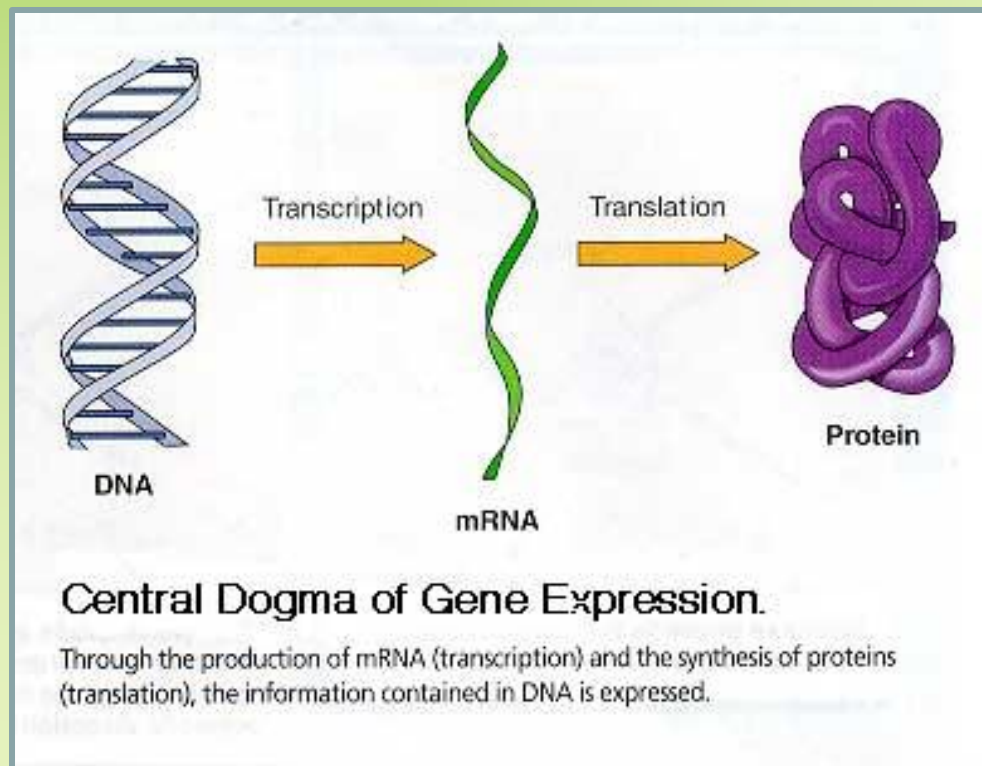
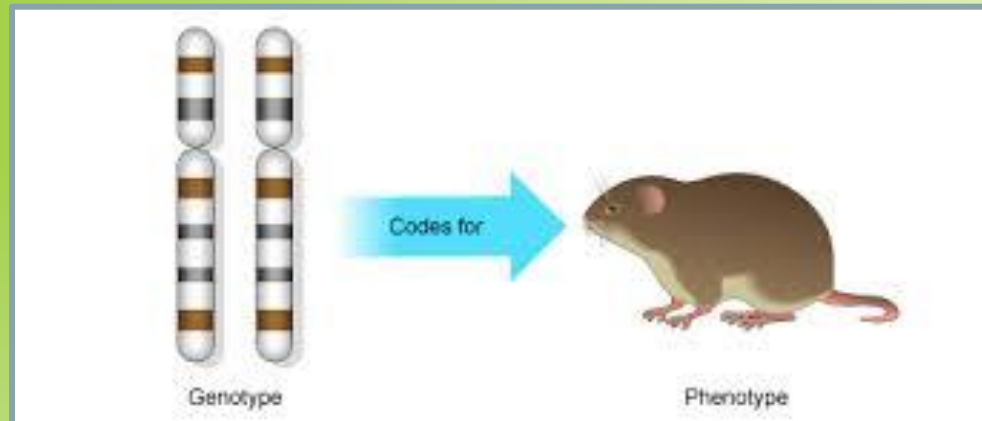
- I. What is epigenetics?
- II. What are the most common epigenetic modifications?
- III. What are the consequences for an individual health/disease?
- IV. How can compounds alter epigenetic processes?

## **2. Epigenomic analyses at Maastricht University**

- I. Techniques for the different epigenomic endpoints
- II. Challenges:
  - III. data analyses
  - IV. Integrative data analyses: iClusterPlus

## **3. Conclusions**

# Central dogma of genetics



# DNA: blue-print is not sufficient



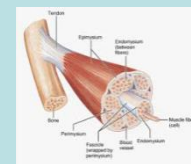
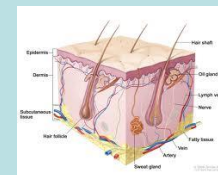
Genetically identical twins:  
Same DNA – yet different  
susceptibility for disease  
(Schizophrenia, cardiovascular  
disease, cancer)



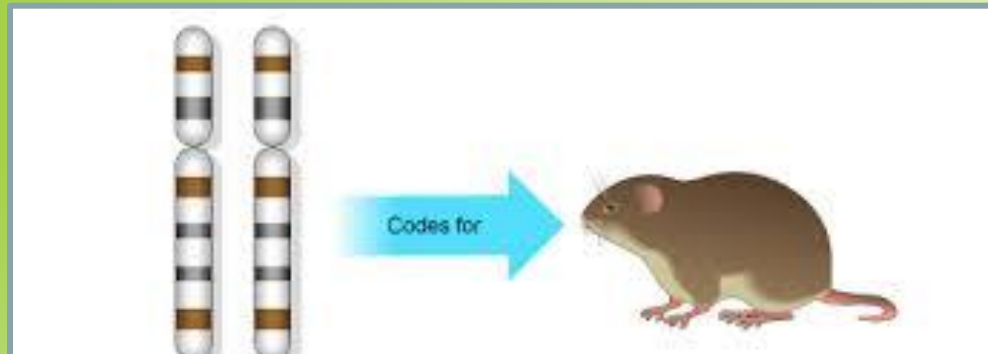
Genetically identical mice:  
Same DNA – yet different  
development  
(obesity, skin color)



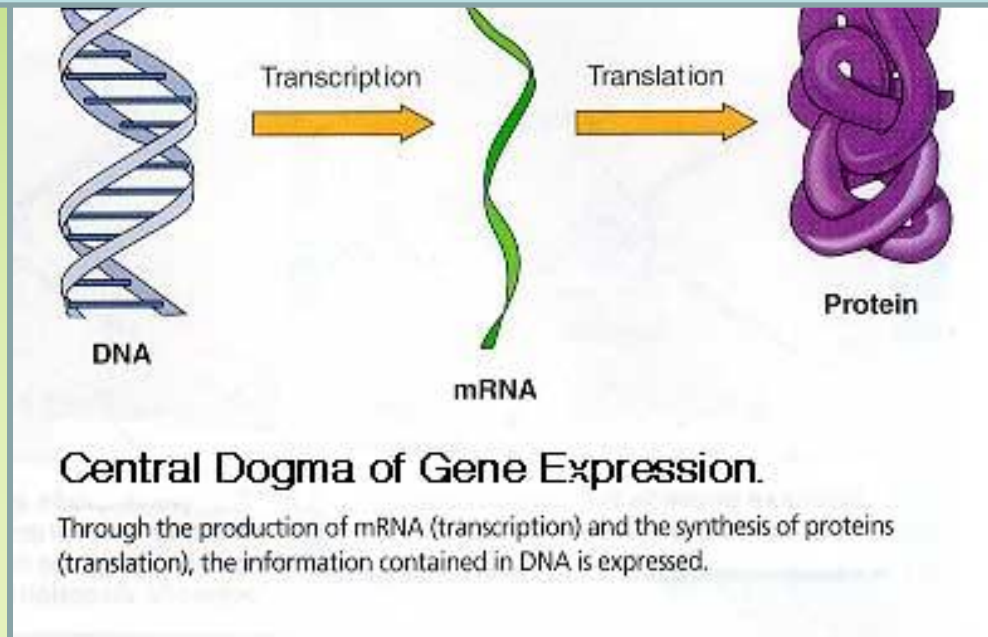
Cell diversity:  
Same DNA – yet different cell  
(skin, muscle, etc.)

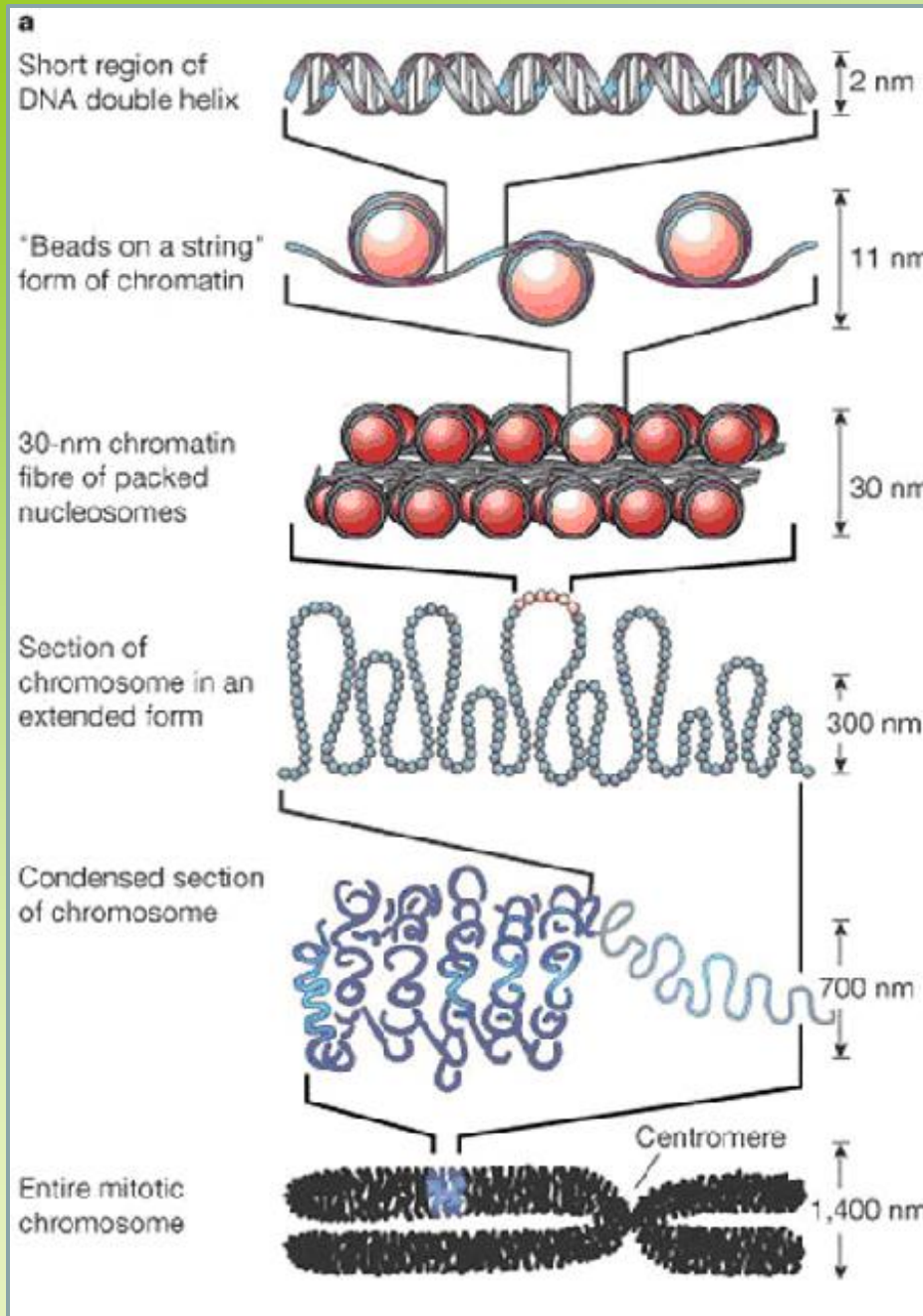


# Central dogma of genetics



Problem: DNA is not naked  
(freely accessible)

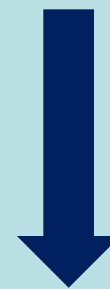




## DNA double helix

Compaction:  
2 meters of DNA  
in nucleus

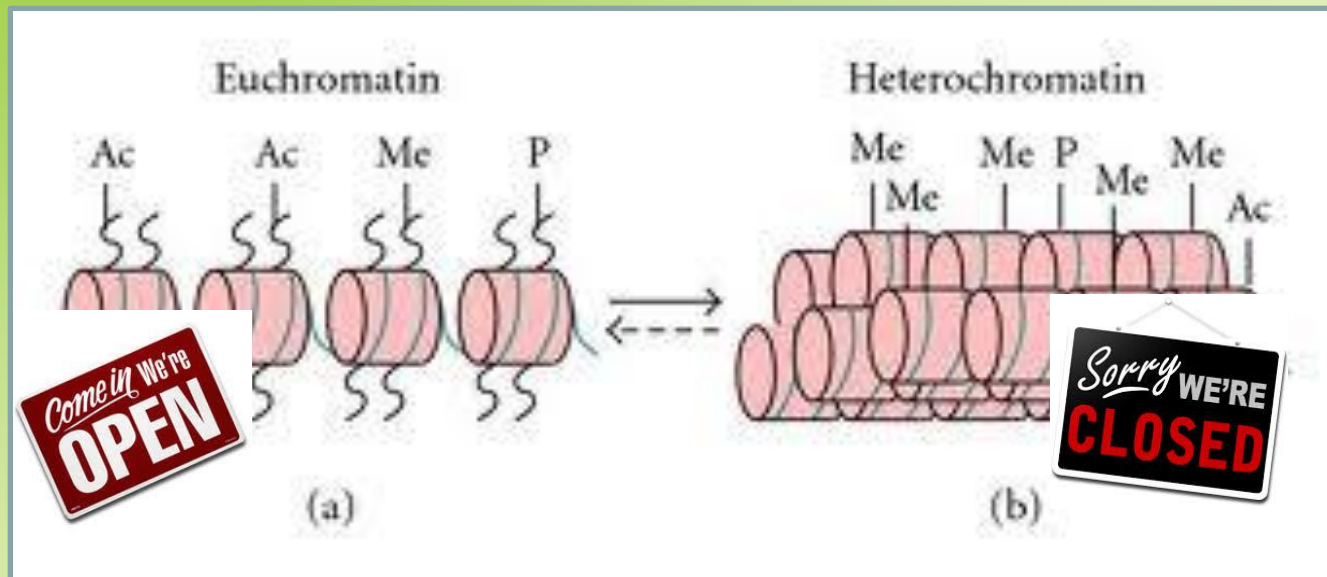
DNA packing  
→ chromatin



DNA not freely  
accessible



# Epigenetics → packing & unpacking of genes



Active DNA

Inactive DNA

# “Upon” the genes = “Epi” genetics

**Epigenetics:** Reversible, heritable changes in **gene function without** a change in DNA sequence

Hardware  
of life



Genetics



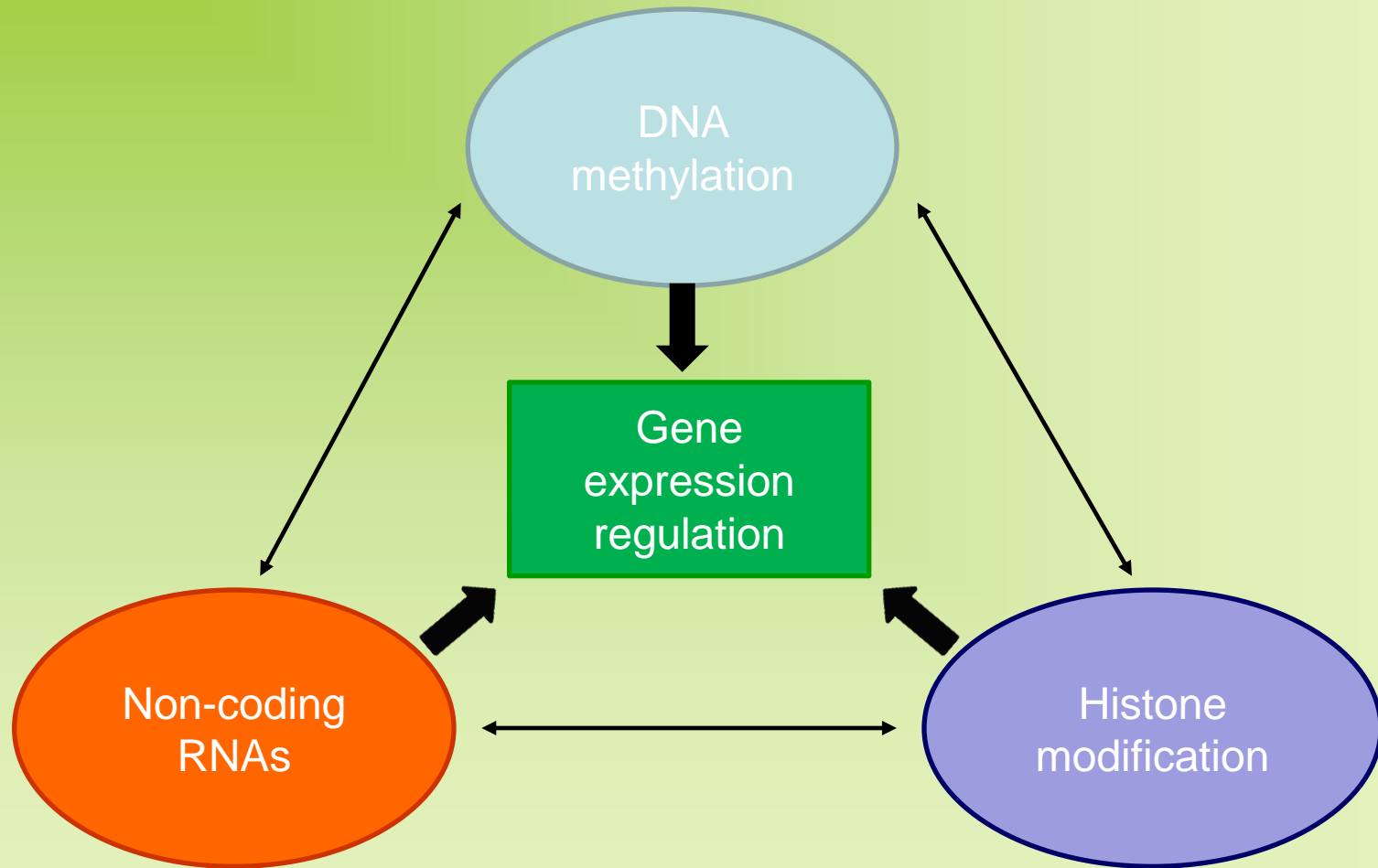
Software  
→ Determines “behavior”  
of hardware  
→ Can be rewritten



Epigenetics

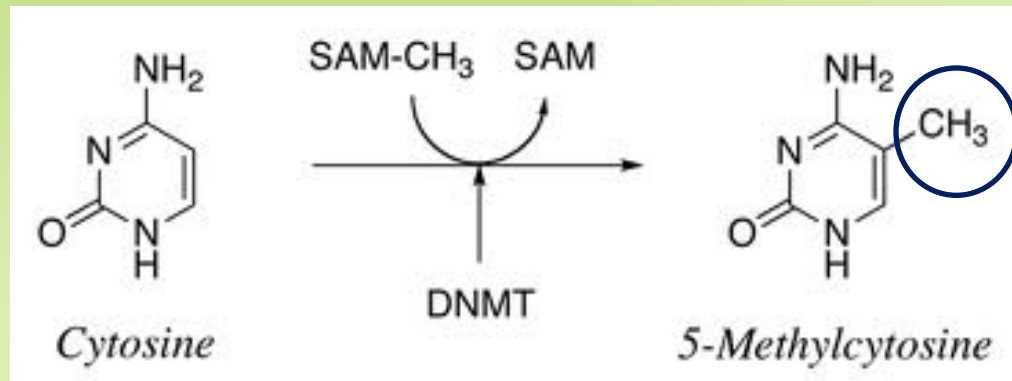


# What are the most common epigenetic modification?



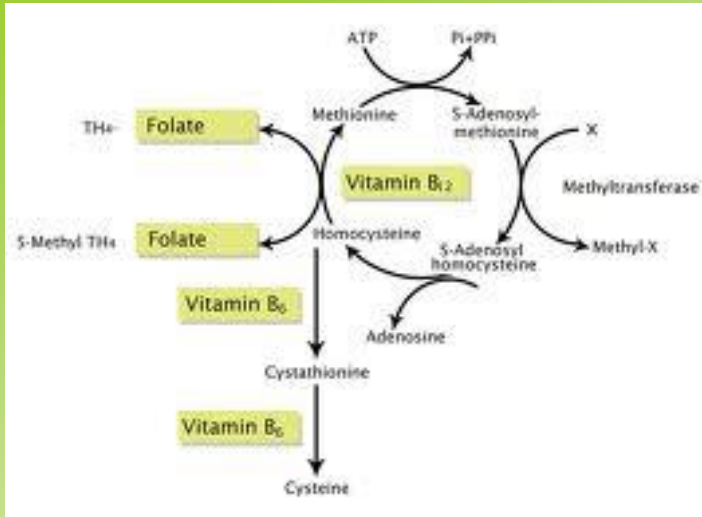
# DNA methylation

- Location: carbon-5 position of cytosine residues → **C**ytosine preceding a **G**uanine
- Mainly in the context of CpG dinucleotides (p → phosphodiester bond)
- 50% of all genes contain CpG-rich stretches of DNA in the promoter region: CpG islands



# DNA methylation

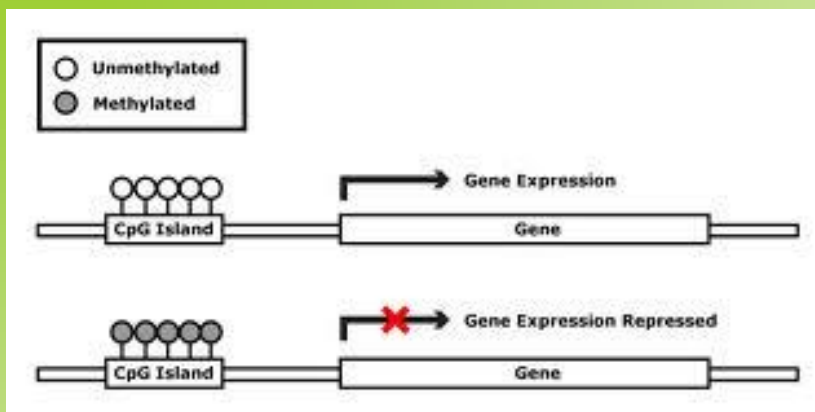
Folic acid:  
important methyl donor in our diet



DNA methyltransferases:

- DNMT1: remains the existing methylation pattern following DNA replication
- DNMT3A and DNMT3B: the novo enzymes that target previously unmethylated CpGs

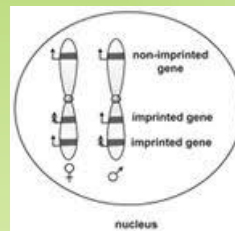
# DNA methylation: physiological roles



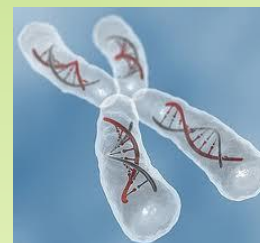
**Gene silencing**

## Examples:

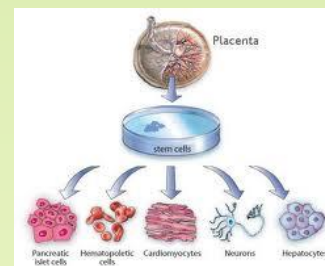
1) Before fertilization  
→ Genomic imprinting



2) During early embryonic development  
→ X-chromosome inactivation



3) Stem cell differentiation  
→ Tissue specific gene expression

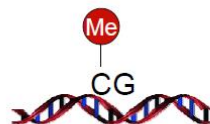


# DNA methylation: pathophysiological roles

## Spina Bifida

Folic Acid: methyl-donor

Important for DNA methylation



## Obesity

### ORIGINAL ARTICLE

Promoter methylation of serotonin transporter gene is associated with obesity measures: a monozygotic twin study

J. Zhao<sup>1</sup>, J. Goldstone<sup>2,3</sup>

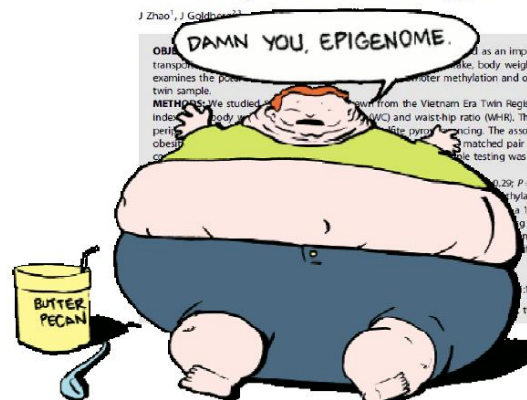
**OBESITY** is an important factor for obesity. The serotonin transporter (5-HTT) gene is involved in body weight and energy balance. This study examines the potential role of promoter methylation and obesity measures in a monozygotic (MZ) twin sample.

**METHODS:** We studied 100 monozygotic twins from the Vietnam Era Twin Registry. Obesity measures include body mass index (BMI), waist circumference (WC) and waist-hip ratio (WHR). The 5-HTT promoter methylation profile in peripheral blood was determined using a methylation-specific PCR assay. The association between methylation variation and obesity measures was examined using a matched pair analysis, adjusting for age, smoking, alcohol consumption and other potential confounders. Multiple testing was controlled using the adjusted false discovery rate.

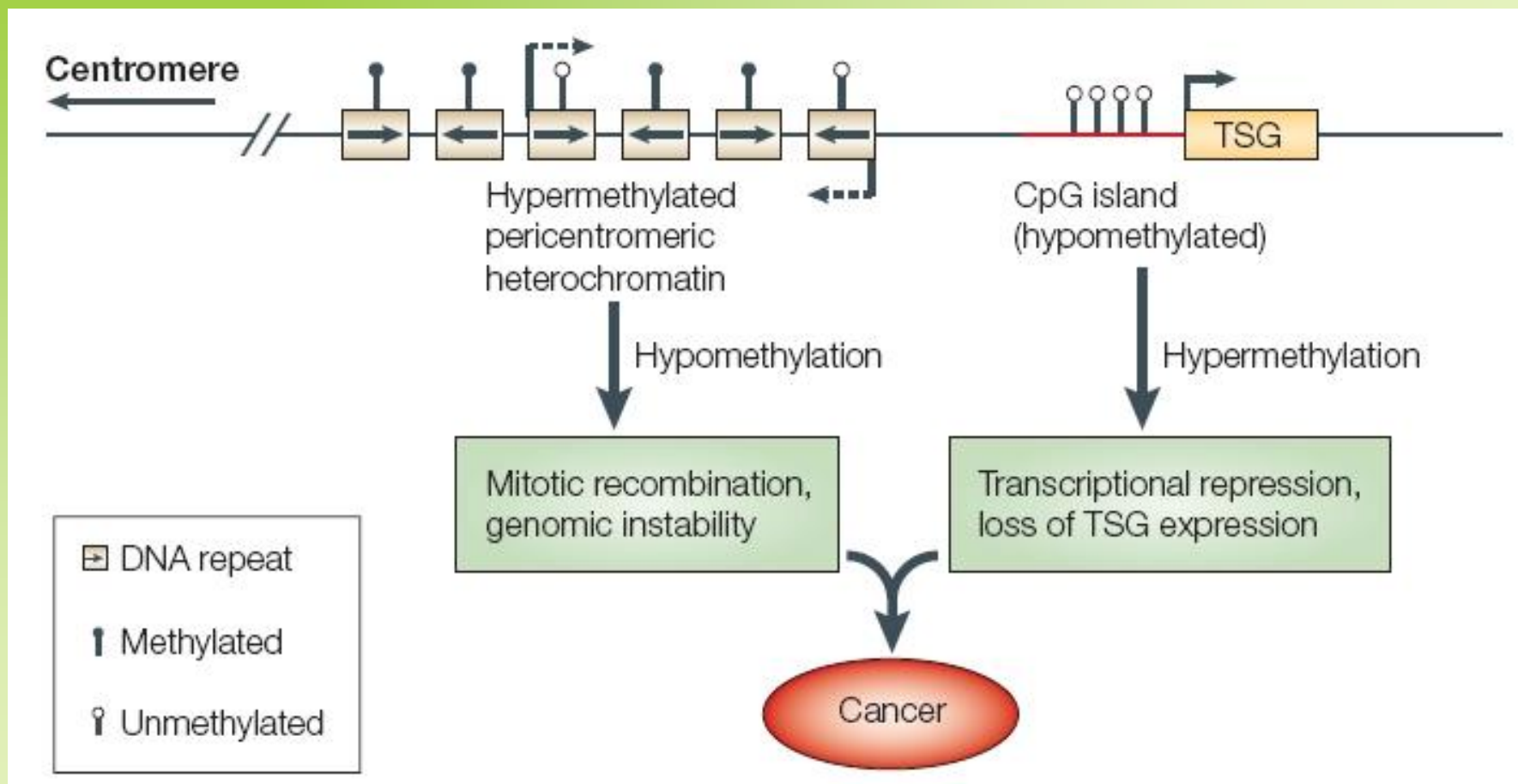
**RESULTS:** A 1% increase in mean methylation was associated with a 0.29% increase in BMI ( $r = 0.29$ ;  $P = 0.002$ ), body weight ( $r = 0.31$ ;  $P < 0.0001$ ) and waist circumference ( $r = 0.28$ ;  $P = 0.002$ ). These associations were significantly correlated with the increase in body weight (95% CI 0.16–2.16;  $P = 0.002$ ), waist circumference (95% CI 0.16–2.16;  $P = 0.002$ ) and WHR (95% CI 0.16–2.16;  $P = 0.002$ ) after adjusting for potential confounders.

**CONCLUSIONS:** These findings suggest that promoter methylation of the 5-HTT gene is associated with an increased prevalence of obesity within monozygotic twins.

10.1038/gj.2012.8  
twins



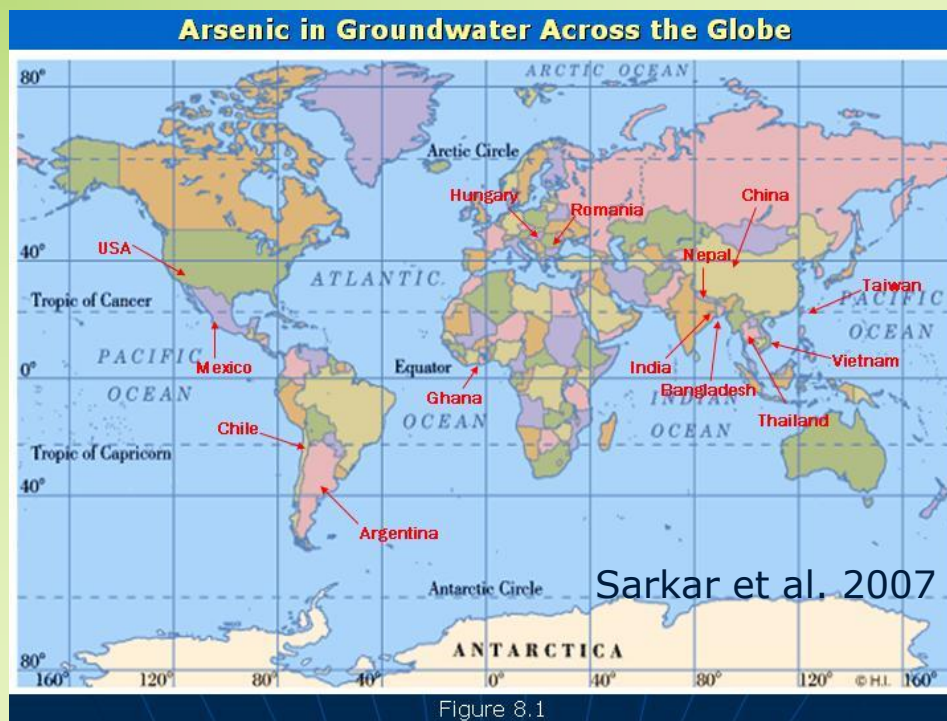
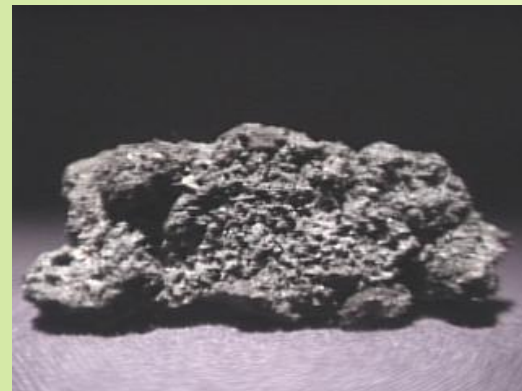
# DNA methylation: pathophysiological roles





## Arsenic

- Grey arsenic;
- Present in minerals and in native state;
- Agricultural use;
- Food additive;
- Medical use;
- Exposure:  
oral (drinking water)  
& inhalation.



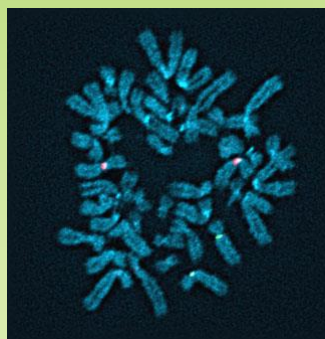
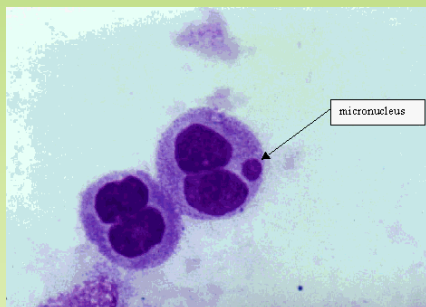
# Arsenic

- Toxic metalloid;
- Group I carcinogen (IARC): associated with lung, bladder, liver, and nonmelanoma skin cancers;
- other diseases like skin lesions, vascular diseases, reproductive toxicity, neurological effects.



# Arsenic: proposed mechanisms for tumorigenesis

- oxidative stress;
- co-carcinogenesis with other environmental toxicants;
- genotoxic damage and chromosomal abnormalities (micronuclei, chromosomal instability);



- No induction of point mutations!
- Indirect effect on DNA? → epigenetic modifications?
- As → epigenetic carcinogen?

# Arsenic: induction of hypo- and hypermethylation

**Table 1.** Arsenic exposure and global DNA methylation.

Model	Arsenical	Dose	Time (weeks)	Global DNA methylation	References
<b>Human cells</b>					
Prostate epithelial cell line RWPE-1	As <sup>III</sup>	5 µM	16	Hypo	Coppin et al. 2008
Prostate epithelial cell line RWPE-1	As <sup>III</sup>	5 µM	29	Hypo	Benbrahim-Tallaa et al. 2005
HaCaT keratinocytes	As <sup>III</sup>	0.2 µM	4	Hypo	Reichard et al. 2007
<b>Animal cells</b>					
TRL 1215 rat liver epithelial cell line	As <sup>III</sup>	125–500 nM	18	Hypo	Zhao et al. 1997
V79-C13 Chinese hamster cells	As <sup>III</sup>	10 µM	8	Hypo	Sciandrello et al. 2004
<b>Animal studies</b>					
Goldfish	As <sup>III</sup>	200 µM	1	Hypo	Bagnyukova et al. 2007
Fisher 344 rat	As <sup>III</sup>	50 µg/g body weight	12	Hypo	Uthus and Davis 2005
129/SvJ mice	As <sup>III</sup>	45 ppm	49	Hypo	Chen et al. 2004
C3H mice	As <sup>III</sup>	85 ppm	1.5	Hypo	Waalikes et al. 2004
C57BL/6J mice	As <sup>III</sup>	2.6–14.6 µg/g body weight	18.5	Hypo	Okoji et al. 2002
Homozygous Tg.AC mice	As <sup>III</sup>	150 ppm	17	Hypo	Xie et al. 2004
	As <sup>V</sup>	200 ppm			
	MMA <sup>V</sup>	1,500 ppm			
	DMA <sup>V</sup>	1,200 ppm			
<b>Human subjects</b>					
	As <sup>III</sup>	2–250 µg/L	NA	Hyper	Pilsner et al. 2007; Majumdar et al. 2010
	As <sup>III</sup>	2–250 µg/L	NA	Hypo (in skin lesion patients)	Pilsner et al. 2009

Abbreviations: Hyper, hypermethylated; Hypo, hypomethylated; NA, not available. See text for additional information on human subjects.

**Table 2.** Arsenic exposure and gene-specific (promoter) methylation status.

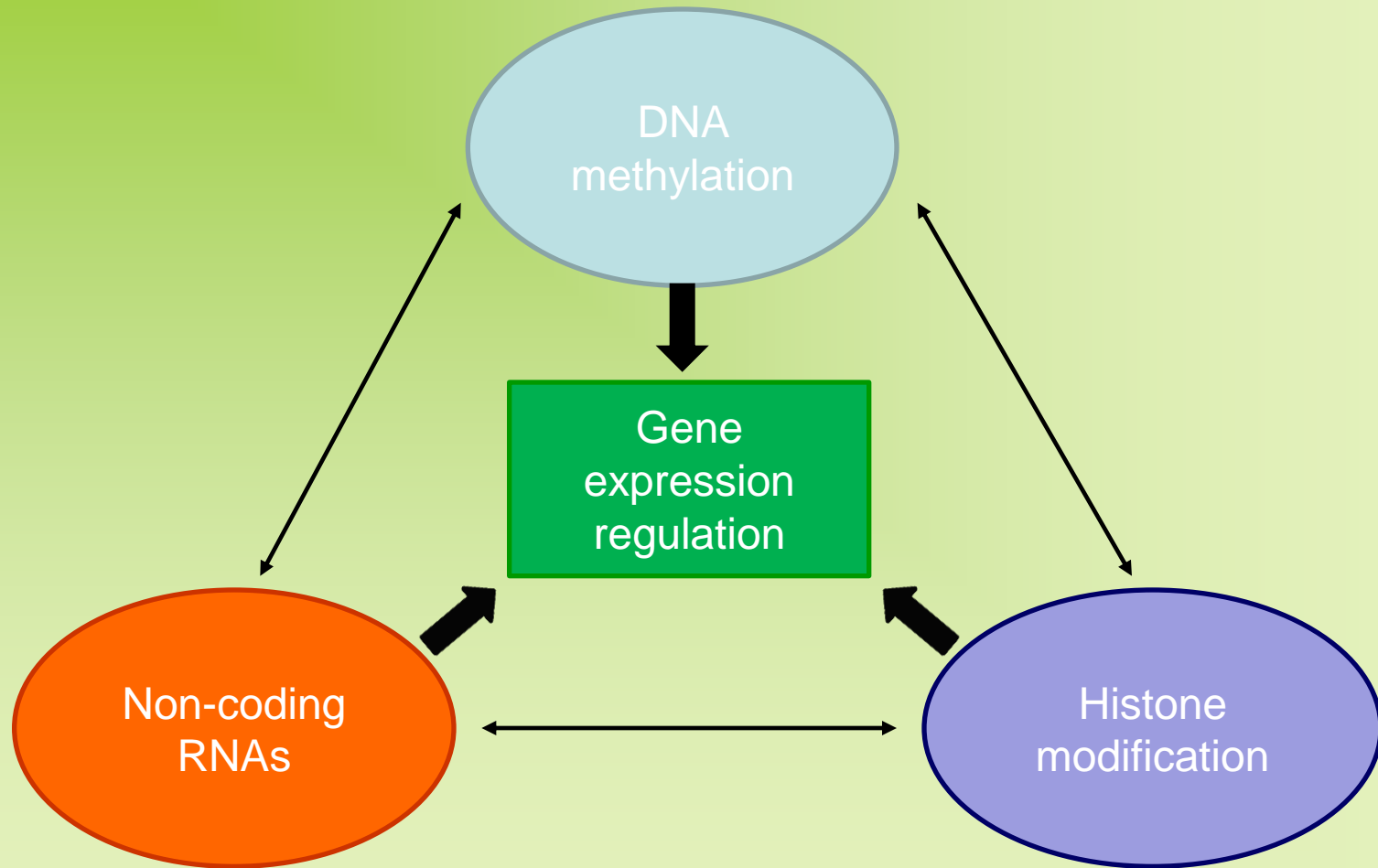
Mode	Arsenical	Dose	Time (weeks)	Genes		Reference
				Hyper	Hypo	
Human cells						
UROtsa urothelial cells	As <sup>III</sup>	1 μM	9	<i>DBC1, FAM83A, ZSCAN12, C10TNF6</i>		Jensen et al. 2008
	MMA <sup>III</sup>	50 nM				
Uroepithelial SV-HUC-1 cells	As <sup>III</sup>	2, 4, 10 μM	24 or 52	<i>DAPK</i>		Chai et al. 2007
Myeloma cell line U266	As <sup>III</sup>	1, 2 μM	0.4	<i>P16</i>		Fu and Shen 2005
Lung adenocarcinoma A549 cells	As <sup>III</sup>	0.08–2 μM	0.3	<i>P53</i>		Mass and Wang 1997
	As <sup>V</sup>	30–300 μM	0.3			
Animal cells						
Syrian hamster embryo cells	As <sup>III</sup>	3–10 μM	0.3		<i>c-myc, c-Ha-ras</i>	Takahashi et al. 2002
	As <sup>V</sup>	50–150 μM	0.3			
TRL 1215 rat liver epithelial cells	As <sup>III</sup>	125–500 nM	8 or 18		<i>c-myc</i>	Chen et al. 2001
Animal studies						
C57BL/6J mice	As <sup>III</sup>	2.6–14.6 μg/g body weight	18.5	<i>p16, RASSF1</i>	<i>c-Ha-ras</i>	Okoji et al. 2002
A/J mice	As <sup>V</sup>	100 ppm	74			Cui et al. 2006a
C3H mice	As <sup>III</sup>	85 ppm	1.4			Waalikes et al. 2004
Human subjects						
	As <sup>III</sup>	NA	NA	<i>DAPK</i>		Chen et al. 2007
	As <sup>III</sup>	Variable <sup>a</sup>	NA	<i>p53, P16</i>		Chanda et al. 2006
	As <sup>III</sup>	NA	NA	<i>p16</i>		Zhang et al. 2007b
	As <sup>III</sup>	Variable <sup>b</sup>	NA	<i>RASSF1A, PRSS3</i>		Marsit et al. 2006b

Abbreviations: ERα, estrogen receptor α; Hyper, hypermethylated; Hypo, hypomethylated; NA, not available.

<sup>a</sup>Study subjects were grouped based on historical arsenic concentration in drinking water, and the range of arsenic concentration in drinking water was < 50 µg/L to > 300 µg/L. <sup>b</sup>The estimated toenail arsenic concentration of study subjects was < 0.01 µg/L to > 50 µg/L.

Ren et al. Environ Health Perspect 119:11–19 (2011)

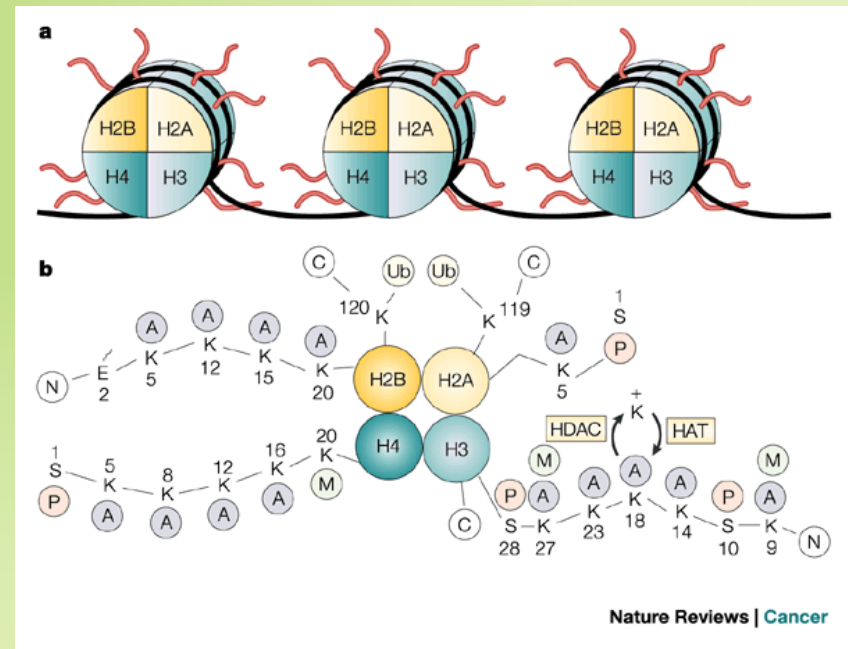
# What are the most common epigenetic modification?



# Histone modifications: “histone code”

## → Chemical modification of tails

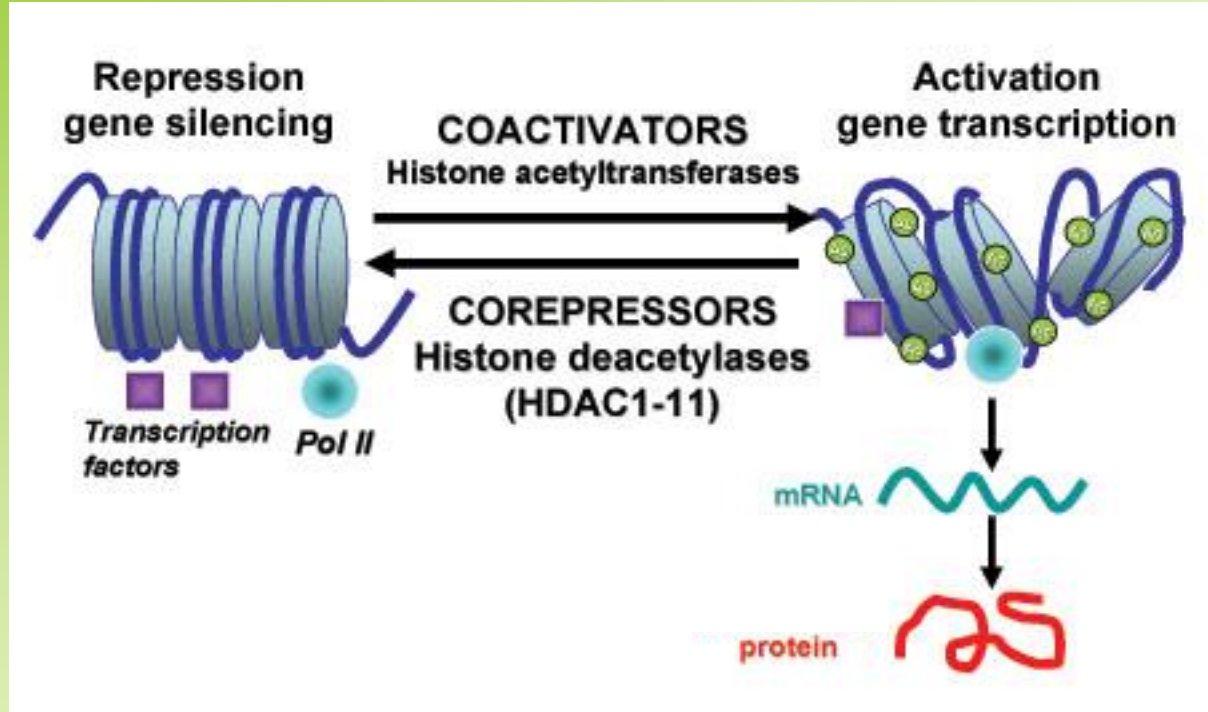
- Chromatin is built up out of nucleosomes
- A nucleosome consist of histone octamer + DNA
- A histone octamer contains 2 copies of each of the histones: H2A, H2B, H3 and H4
- Histones have a histone tail that protrudes from the nucleosomes
- The histone tail is frequently post-translationally modified
- Histone modifications include **acetylation, methylation, phosphorylation** and ubiquitination





# Histone modifications

## → Histone (de)acetylation



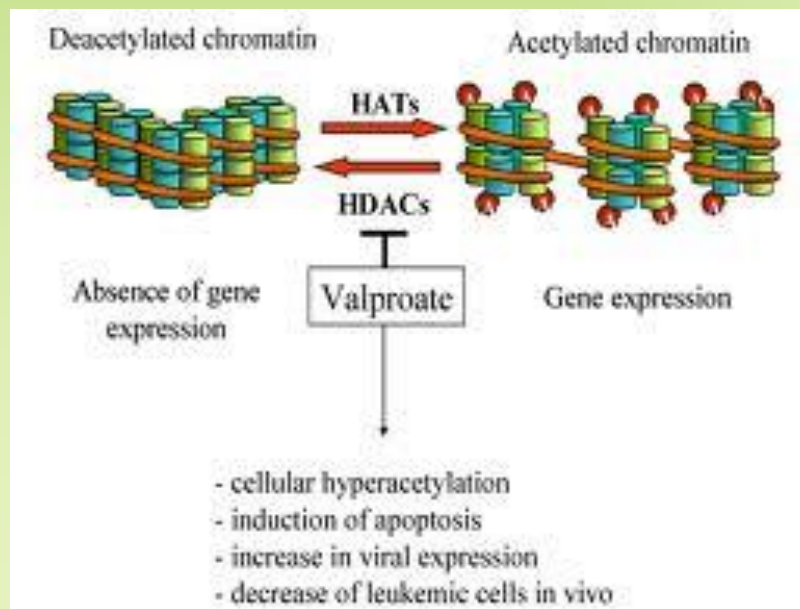
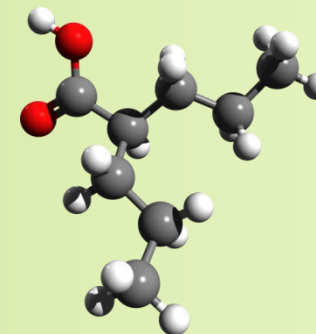
# Example: Compound induced histone acetylation changes

## Valproic acid:

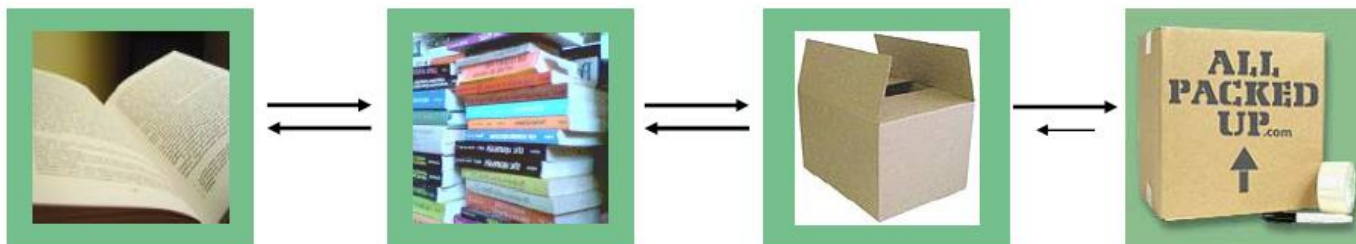
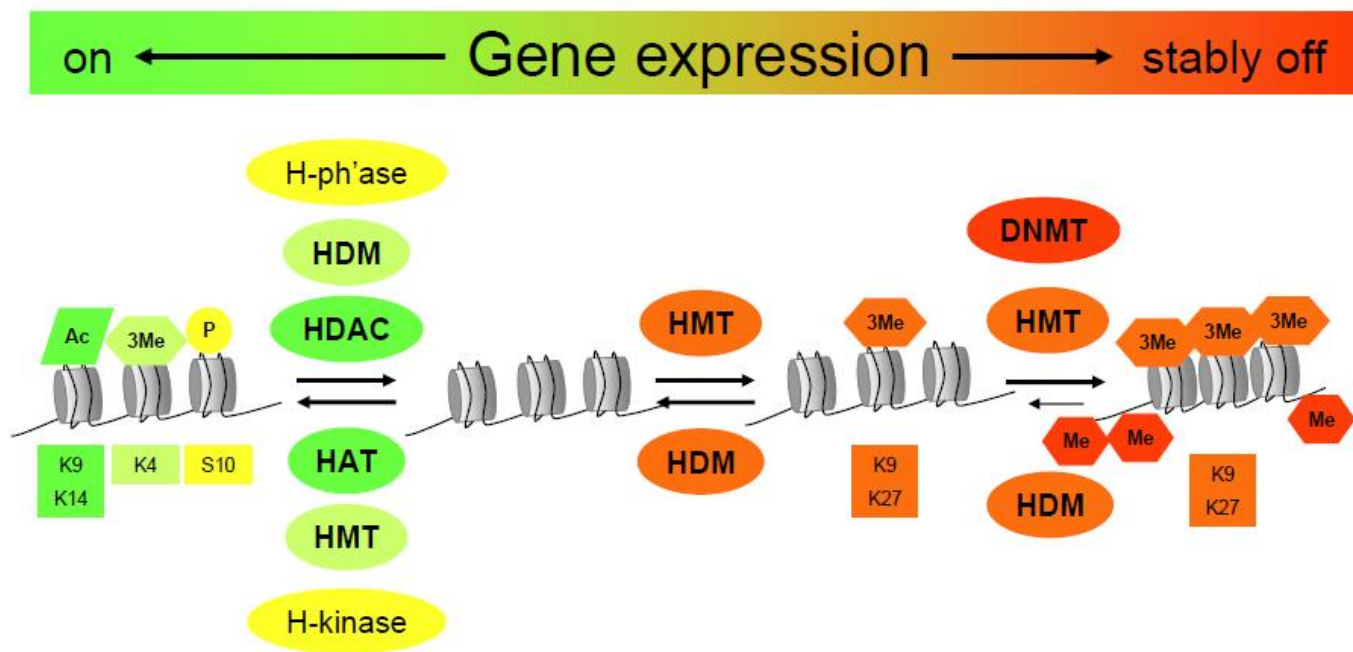
- Clinical use as an anticonvulsant and mood-stabilizing drug
- Side effects: liver damage in patients

## Epigenetic effect:

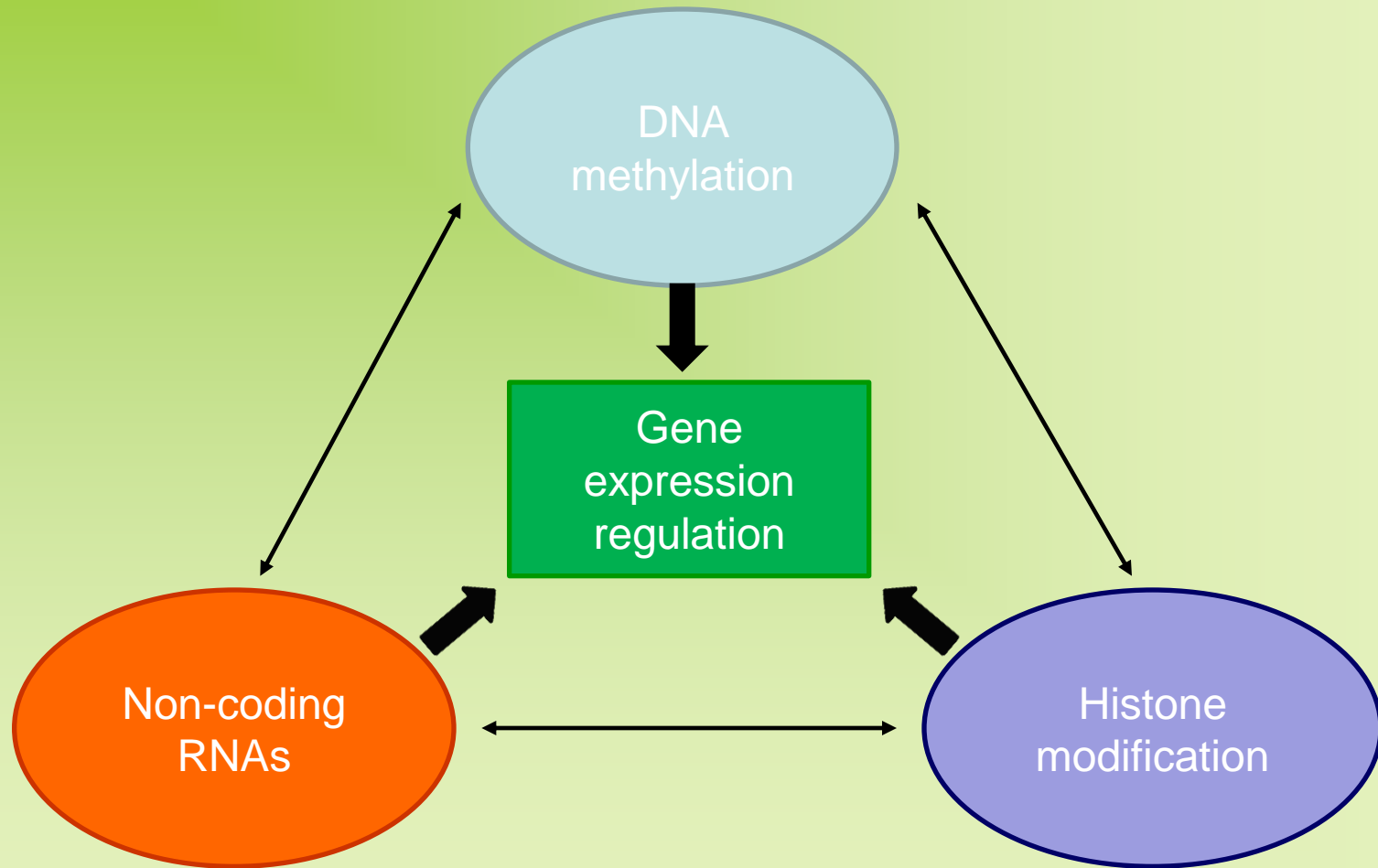
- inhibits the enzyme histone deacetylase 1, thereby inducing histone hyperacetylation
- stimulates active demethylation in a replication independent manner by increasing accessibility of demethylase enzyme



# Epigenetics → packing & unpacking of genes: interplay between DM and HA

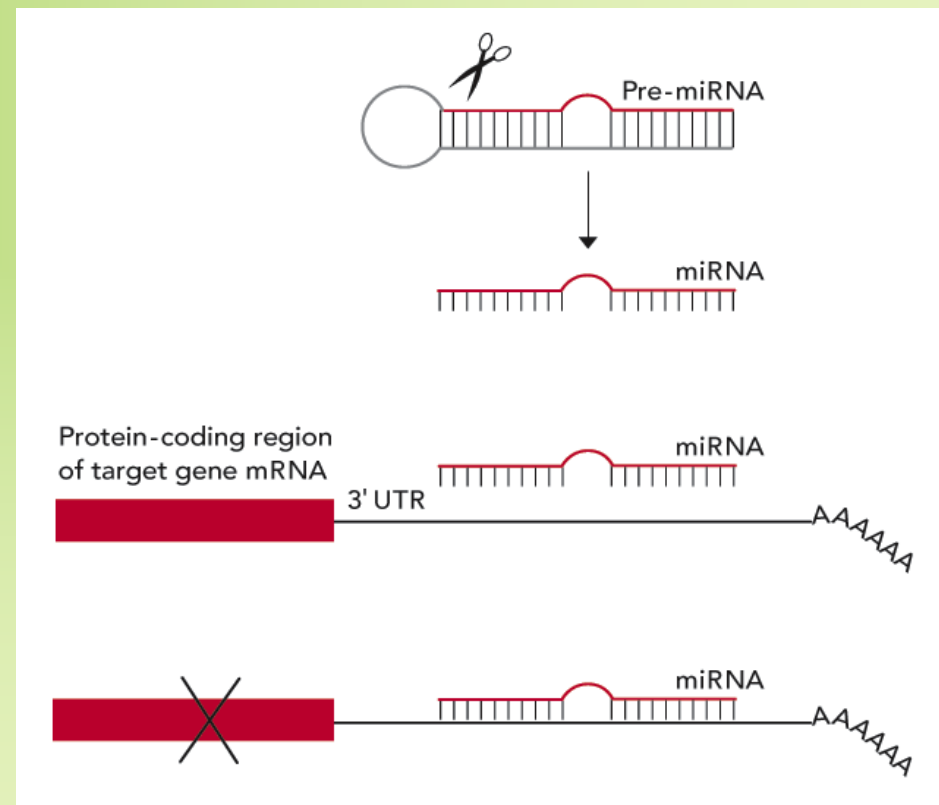


# What are the most common epigenetic modification?

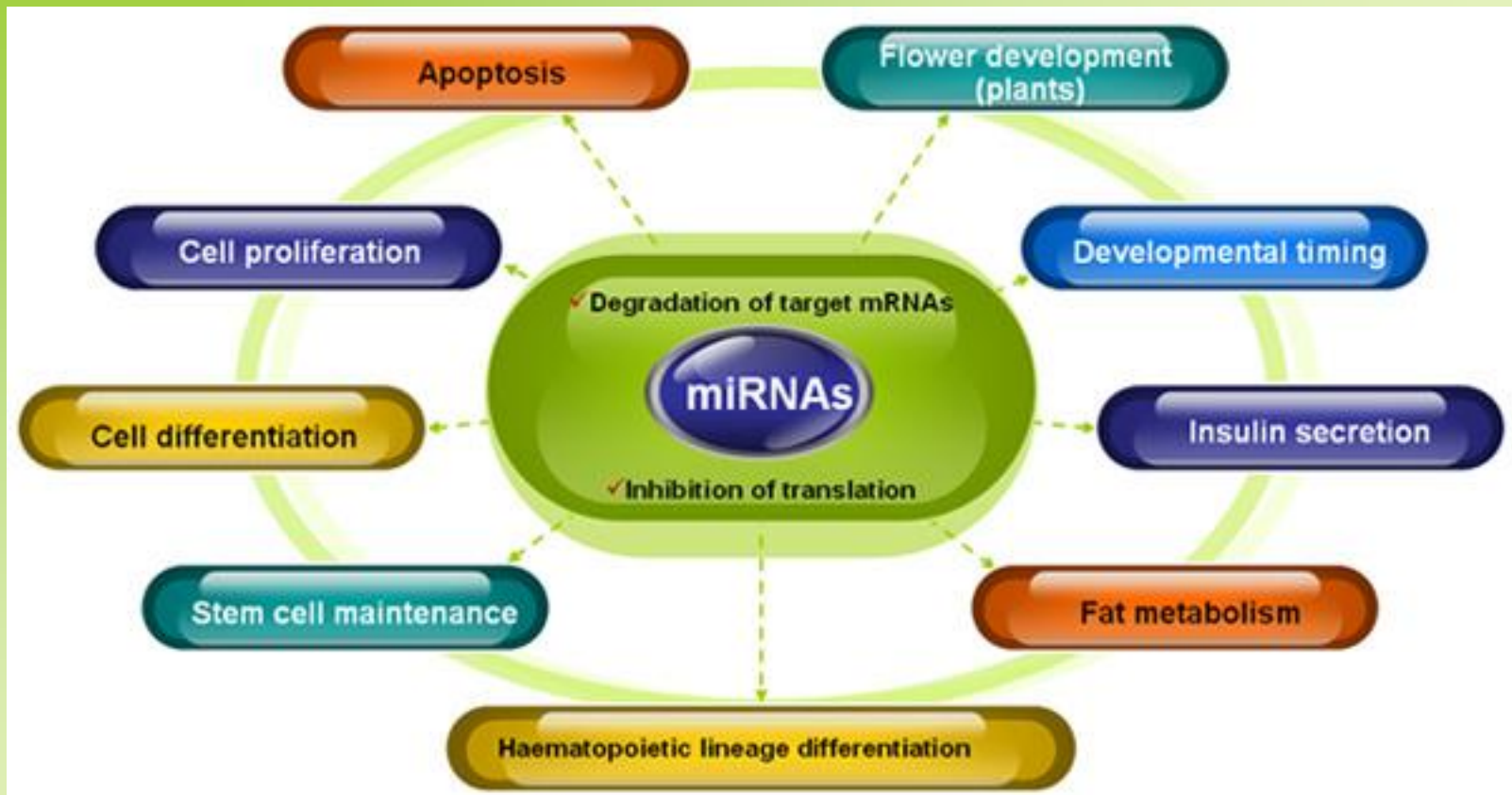


# Non-coding RNA's: microRNA's

- Long non coding RNAs: microRNA: 22 nucleotides long
- Single stranded
- Functions in transcriptional and post-transcriptional regulation of gene expression
- Encoded by eukaryotic nuclear DNA
- miRNAs function via base-pairing with complementary sequences within mRNA molecules, usually resulting in gene silencing via translational repression or target degradation.
- The human genome may encode over 1000 miRNAs, which may target about 60% of mammalian genes
- Abundant in many human cell types
- Different sets of expressed miRNAs are found in different cell types and tissues



# microRNA's: physiological roles





# microRNA's: Toxicological roles

Toxicology Letters 198 (2010) 100–105



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**Toxicology Letters**

journal homepage: [www.elsevier.com/locate/toxlet](http://www.elsevier.com/locate/toxlet)



Mini review

## MicroRNAs and their implications in toxicological research

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<sup>b</sup> Cell Culture and High Throughput Screening Core Facility, Department of Biological Sciences, University of Texas at El Paso, El Paso, TX 79968, United States

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### ABSTRACT

MicroRNAs (miRNAs) are non-coding regulatory RNA molecules that bind target messenger RNAs (mRNA) and suppress their translation into proteins. When an organism is exposed to a toxic compound, cells respond by altering the pattern of gene expression, including miRNAs. Altered miRNA expression affects protein translation, which in turn alters cellular physiology causing adverse biological effects. Moreover, different types of cellular stress have been shown to affect miRNA expression as a mechanism of adaptation or tolerance to stress factors in order to survive. Besides an updated theoretical background concerning miRNAs biology, biogenesis, function, and roles in disease; this mini review provides an overview of miRNAs response to exogenous agents such as environmental stressors and toxic compounds. The implications of miRNAs in toxicogenomics as well as the new avenues of research of miRNAs in toxicology are discussed.

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# microRNA's: Toxicological roles

**Table 1**  
miRNA and cellular stress.

Organism	Stressor	miRNA	Target	Biologic effect	Reference
Plants	Oxidative stress	↓miR-398	Cu/Zn superoxide dismutases	Superoxide radicals detoxification	Sunkar et al. (2006)
	Drought	↑miR-159	MYB transcription factors	Growth and development arrest	Reyes and Chua (2007)
	Bacterial infection	↑miR-393	F-box auxin receptors: TIR1, AFB2, and AFB3	Bacteria growth restriction	Navarro et al. (2006)
	Phosphate (Pi) starvation	↑miR-399	Ubiquitin-conjugating enzyme (UBC)	Attenuation of primary-root elongation	Fujii et al. (2005)
	Sulfate deprivation	↑miR-395	Sulfate transporter AST68 and ATP sulfurylases	Sulfur homeostasis	Sunkar et al. (2007)
	Heavy metal stress	↓miR-398	Cu/Zn-superoxide dismutases (CSD1 and CSD2)	Superoxide radicals detoxification	Ding and Zhu (2009)
	Heavy metal stress	↑miR-393	E3 ubiquitin ligase; TIR1 (transport inhibitor response1)	Auxin signaling down-regulation; Less proteolysis of E3 ubiquitin ligase targeting proteins	Ding and Zhu (2009)
	Heavy metal stress	↑miR-171	Transcription factors that regulate floral development	Cadmium tolerance	Ding and Zhu (2009)
Animals	Folate deprivation	↑miR-222	S-adenosyl methionine, methyl-group donor for cellular methylation	Epigenetic alterations	Marsit et al. (2006)
	Hypoxia	↓miR-15b, -16, -20b	VEGF (vascular endothelial growth factor) and other angiogenic factors	Angiogenesis regulation	Hua et al. (2006)
	Hypoxia	↑miR-23, -24, -26	BID, NIX/BNIP3L, caspase-7, BIM, BAK1	Antiapoptotic effect	Kulshreshtha et al. (2007)
	Hypoxia	↑miR-27, -30, -181	APAF1, caspase-3, BIM	Antiapoptotic effect	Kulshreshtha et al. (2007)
	Hypoxia	↑miR-21, -23, -26	cdc25a, cyclin H, cyclin D2, cyclin E1	Cell arrest	Kulshreshtha et al. (2007)
	Hypoxia	↑miR-103, -107	cdc25a	Cell arrest	Kulshreshtha et al. (2007)
	Hypoxia	↑miR-210, -373	DNA repair pathways: homology-dependent repair (HDR) and nucleotide excision repair (NER)	Genetic instability	Crosby et al. (2009)
	Radiation	↑miR-521	DNA repair protein, CSA (Cockayne syndrome protein A)	Sensitization of prostate cancer cells to radiation treatment	Josson et al. (2008)
	Serum starvation (cell culture)	↑miR-369-3	TNFα ARE (tumor necrosis factor alpha AU-rich element)	Translation activation	Vasudevan et al. (2007)

(↑) up-regulation; (↓) down-regulation.

# 2. Epigenomic analyses at Maastricht University:

## Genome-wide analysis of epigenetic actors

### A. DNA methylation analyses

NimbleGen 2.1M Deluxe Promoter Array  
Medip-Chip



Format: [2.1M](#)  
Source: UCSC  
Probe Length: 50-75mer  
Median Probe Spacing: 100bp  
Recommended Storage: Store arrays desiccated at room temperature

Description	Build	Promoter Upstream Tiling (bp)	Promoter Downstream Tiling (bp)	Number of CpG Islands	miRNA Promoters
<b>NEW!</b> Human DNA Methylation 2.1M Deluxe Promoter v2 Array	HG19	8000**	3000	27867	730 (-15kb to mature miRNA)



### B. Histone Acetylation analyses

NimbleGen 2.1M Deluxe Promoter Array  
Chip-Chip



Format: [2.1M](#)  
Source: UCSC  
Probe Length: 50-75mer  
Median Probe Spacing: 100bp  
Recommended Storage: Store arrays desiccated at room temperature

Description	Build	Promoter Upstream Tiling (bp)	Promoter Downstream Tiling (bp)	Number of CpG Islands	miRNA Promoters
<b>NEW!</b> Human DNA Methylation 2.1M Deluxe Promoter v2 Array	HG19	8000**	3000	27867	730 (-15kb to mature miRNA)



### C. miRNA analyses

Agilent Human miRNA Microarray Release 19.0,  
8x60K based on miRBase.  
2006 human miRNAs represented.



➡ Initial raw data analyses: →  
identification of significant genes:  
R-script analyses



Gene lists

# A. DNA methylation

## Overall procedure:

1. DNA isolation plus QC
2. DNA immunoprecipitation (MeDIP) plus QC
3. Microarray processing: labeling, hybridisation, washing, scanning (Roche NimbleGen)
4. Data extraction plus QC
5. Data analyses



## 1. DNA isolation

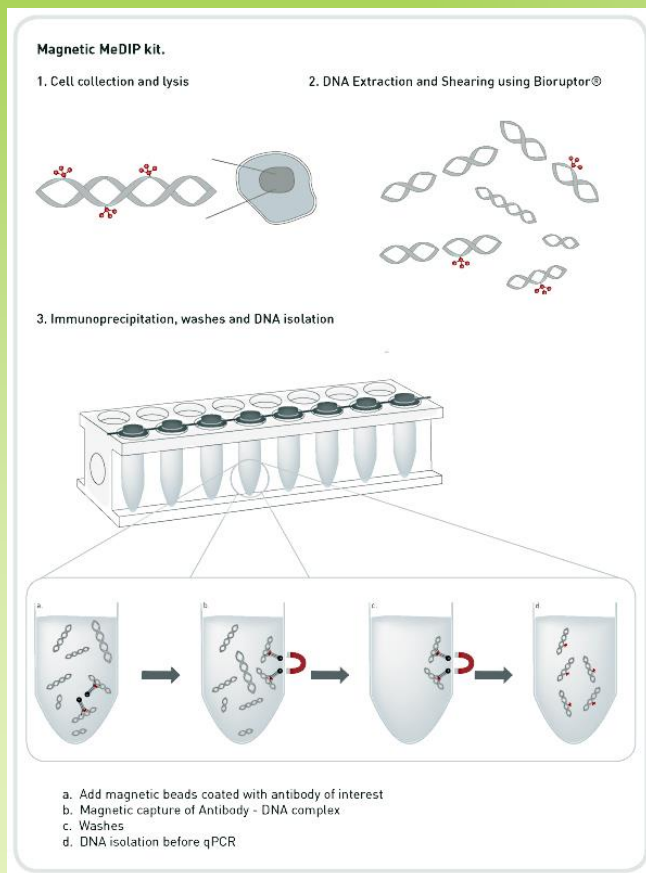
- Cell lysis in digestion buffer plus proteinase K;
- RNase treatment;
- Phenol – Chloroform – Isoamyl alcohol extraction
- Precipitation with NaAc
- Washing with ETOH
- Dissolved in nuclease free water

### QC:

- Nanodrop quantification: spectrum
- 260/280 ratio 1.7 – 1.9
- 260/230 > 1.6

## 2. DNA immunoprecipitation

- Diagenode: MagMeDIP Kit including antibody against 5'-methylcytidine



- Sonication of DNA: 200 – 600 bp
- 1.2  $\mu$ g of sonicated sample used
- Addition of positive and negative controls
- 10% used as input samples (Input)
- Remaining sample immunoprecipitated with antibody against 5'-methylcytidine (MeDIP)
- Using magnetic beads
- Whole genome amplification of both Input and MeDIP samples

### QC

- Determination of methylation enrichment by qPCR ( $\Delta\Delta Cq$ ) of positive and negative controls



### 3. Microarray processing

Roche – NimbleGen  
Human DNA methylation 2.1M Deluxe  
promoter V2 array

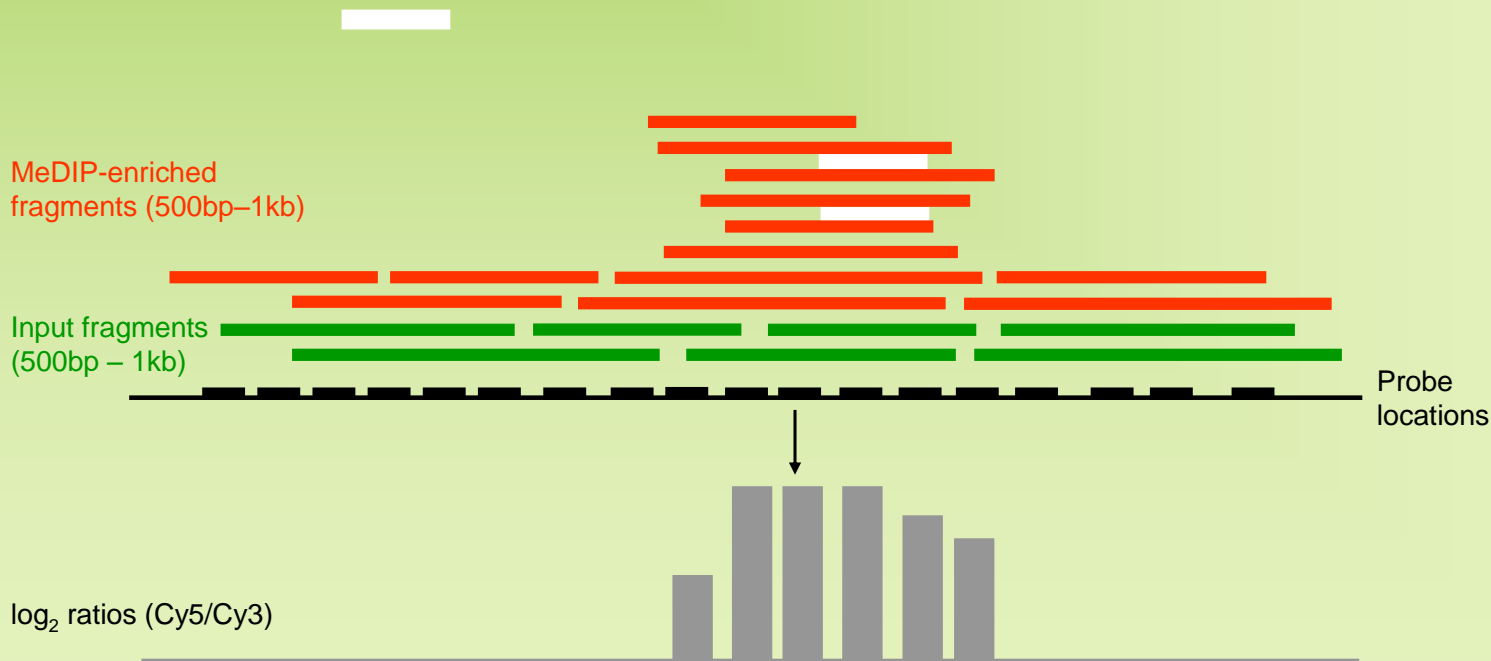
 <b>Format:</b> 2.1M <b>Source:</b> UCSC <b>Probe Length:</b> 50-75mer <b>Median Probe Spacing:</b> 100bp <b>Recommended Storage:</b> Store arrays desiccated at room temperature					
Description	Build	Promoter Upstream Tiling (bp)	Promoter Downstream Tiling (bp)	Number of CpG Islands	miRNA Promoters
<b>NEW!</b> Human DNA Methylation 2.1M Deluxe Promoter v2 Array	HG19	8000**	3000	27867	730(-15kb to mature miRNA)

- Labeling, hybridisation, washing and scanning according to NimbleGen manuals:
- 1 µg Input sample: Cy3; 1 µg MeDIP sample: Cy5

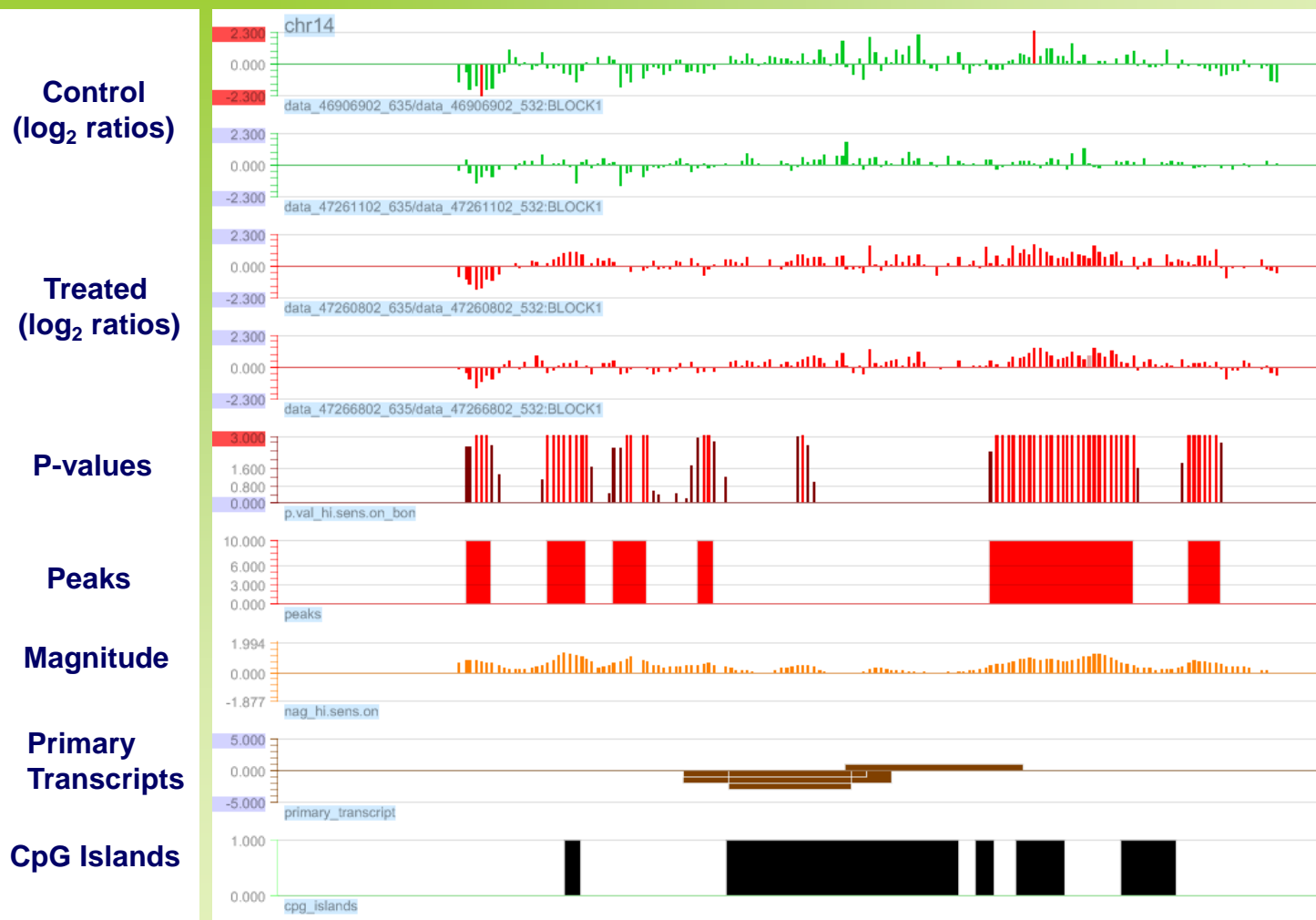


## 4. Data extraction

- NimbleScan software: Generation of raw signal intensities (raw data files)
- BioConductor : R-scripts
  1. T-Quantile normalization on a per channel basis
  2. Calculation of log ratios
  3. QC: image files, boxplots, clustering, correlation plots, MA plots



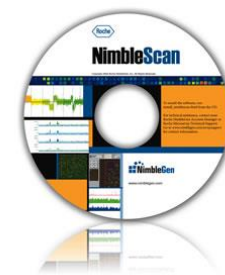
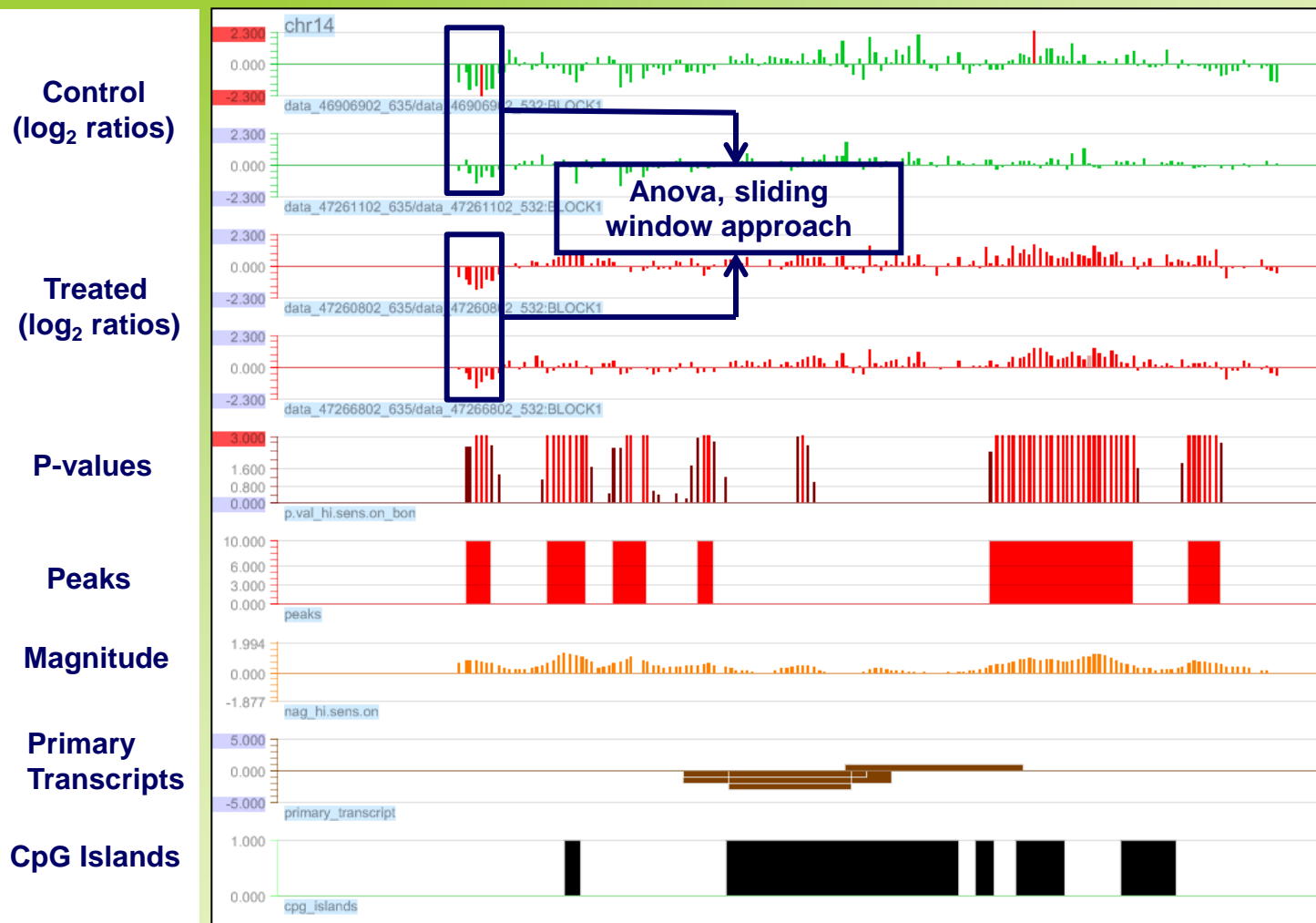
## 5. Data Analyses



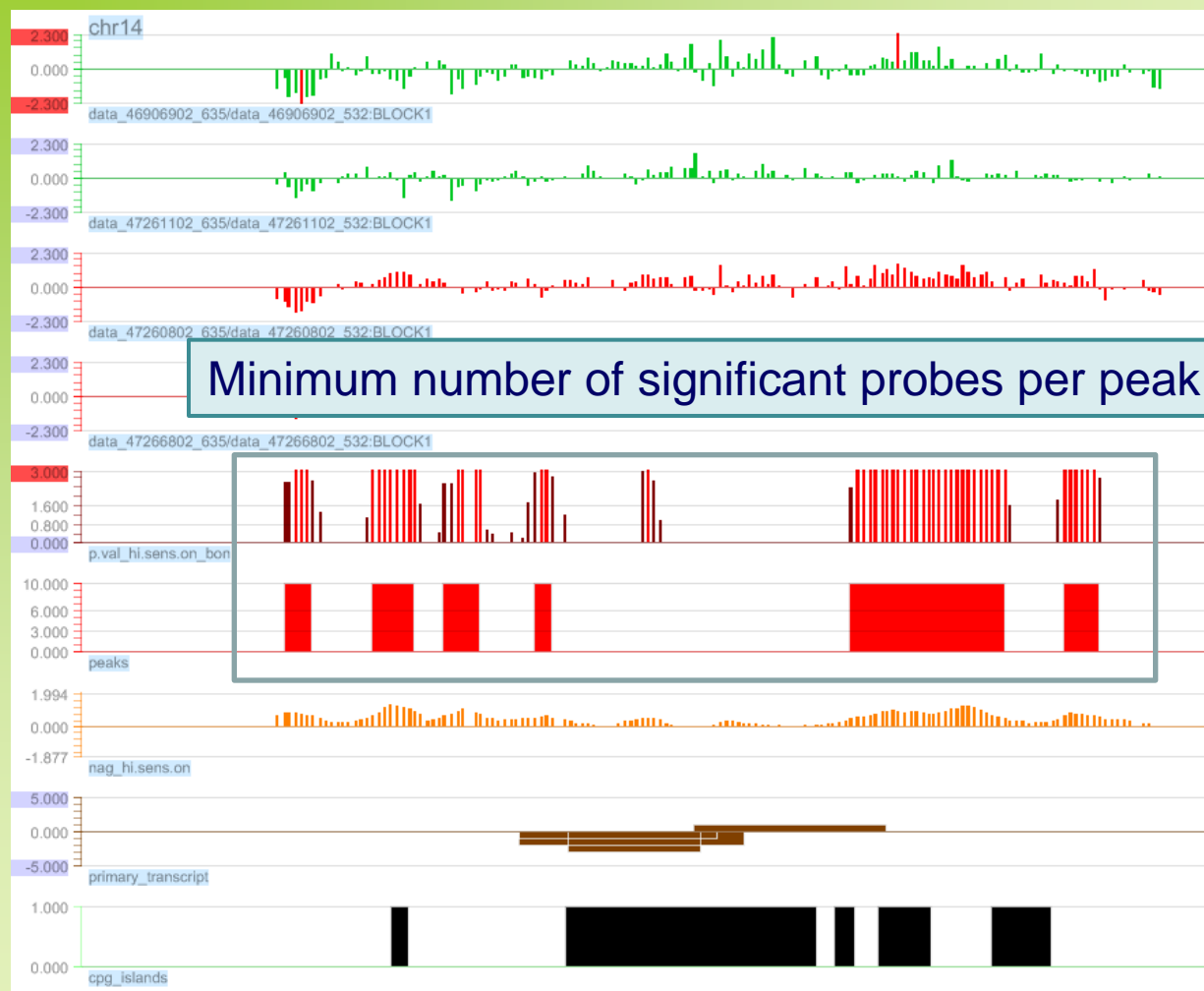
**Anova, sliding window approach**



## 5. Data Analyses



## 5. Data Analyses: define cut-off value



Anova, sliding window approach



## 5. Data Analyses: define cut-off value

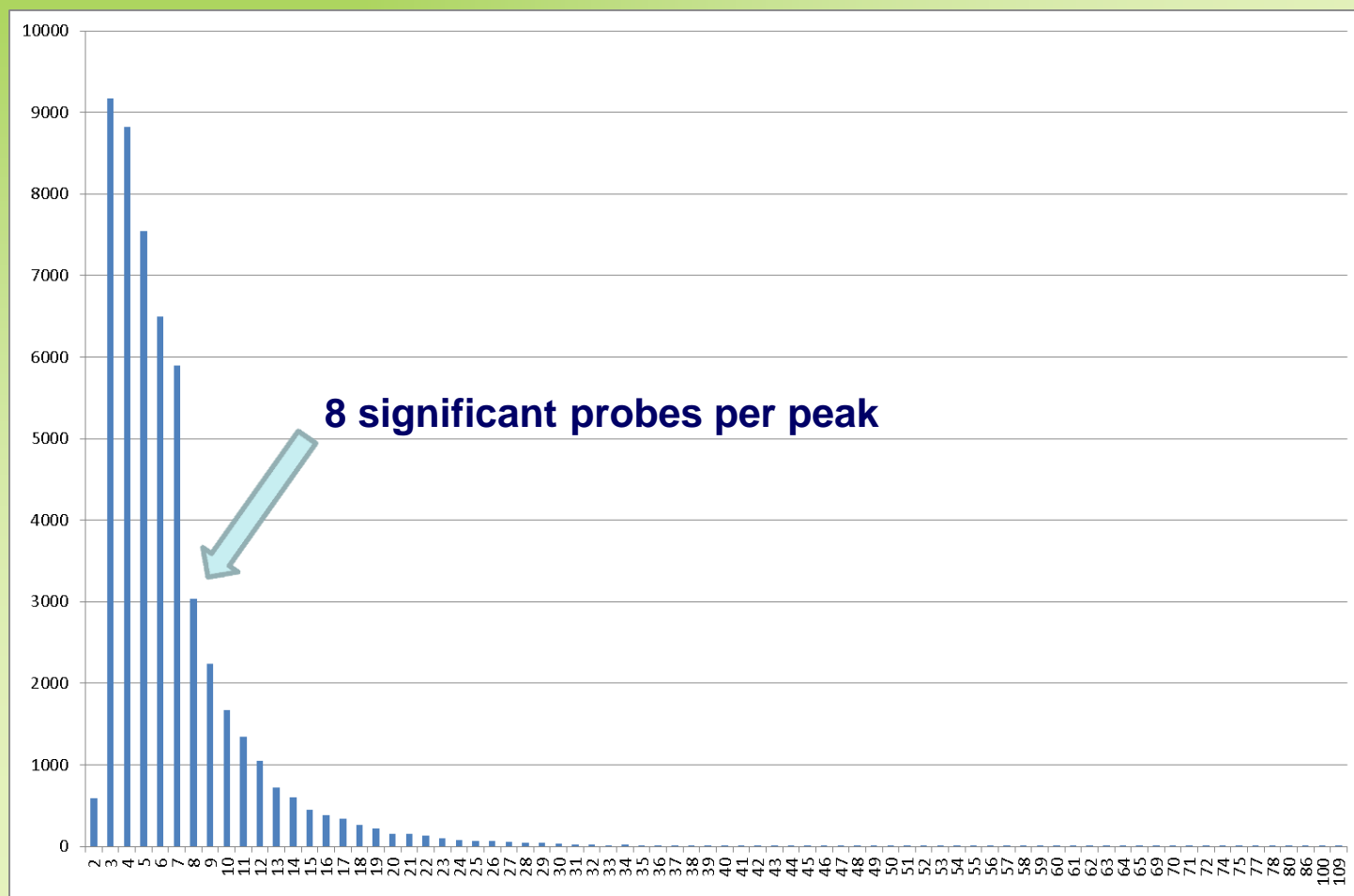
- I. output peak file filtered on significant number of probes per peak
- II. Visualize in histogram
- III. define cutoff value

	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	
1	Original Peaks						Significant probes	probes	ratio		unique ratios	Count		Significant probes	Peak count	
2	chr1	Detect-DMR.R	peaks	17334	17864		3	3	1		0.461538462	1		2	593	
3	chr1	Detect-DMR.R	peaks	21314	21466		2	2	1		0.476190476	1		3	9168	
4	chr1	Detect-DMR.R	peaks	25266	25494		3	3	1		0.505376344	1		4	8827	
5	chr1	Detect-DMR.R	peaks	29086	29284		3	3	1		0.52173913	1		5	7549	
6	chr1	Detect-DMR.R	peaks	43018	43241		3	3	1		0.523809524	2		6	6500	
7	chr1	Detect-DMR.R	peaks	62138	63648		16	16	1		0.529411765	2		7	5894	
8	chr1	Detect-DMR.R	peaks	66639	68333		18	18	1		0.533333333	1		8	3041	
9	chr1	Detect-DMR.R	peaks	69115	70019		10	10	1		0.538461538	3		9	2241	
10	chr1	Detect-DMR.R	peaks	70565	71483		10	10	1		0.555555556	3		10	1671	
11	chr1	Detect-DMR.R	peaks	546061	546609		5	5	1		0.558974359	1		11	1342	
12	chr1	Detect-DMR.R	peaks	566210	570005		39	39	1		0.571428571	3		12	1044	
13	chr1	Detect-DMR.R	peaks	755468	758694		33	33	1		0.577777778	1		13	720	
14	chr1	Detect-DMR.R	peaks	759806	760710		10	10	1		0.583333333	1		14	604	
15	chr1	Detect-DMR.R	peaks	763716	764218		6	6	1		0.588235294	1		15	444	
16	chr1	Detect-DMR.R	peaks	765013	765824		9	9	1		0.6	4		16	386	
17	chr1	Detect-DMR.R	peaks	767614	768036		5	5	1		0.625	5		17	338	
18	chr1	Detect-DMR.R	peaks	811253	811537		4	4	1		0.666666667	5		18	266	
19	chr1	Detect-DMR.R	peaks	813701	814107		5	5	1		0.684210526	1		19	214	
20	chr1	Detect-DMR.R	peaks	814347	815204		8	8	1		0.75	3		20	155	
21	chr1	Detect-DMR.R	peaks	816110	816682		6	6	1		1	51975		21	150	
22	chr1	Detect-DMR.R	peaks	817455	817755		4	4	1					22	137	
23	chr1	Detect-DMR.R	peaks	839709	840404		8	8	1					23	99	



## 5. Data Analyses: define cut-off value

- I. output peak file filtered on significant number of probes per peak
- II. Visualize in histogram
- III. define cutoff value



## 5. Data Analyses: Example of results file

	A	B	C	D	E	F	G	H	I	J
1	NCB_ID	Control 1	Control 2	Control 3	Treated 1	Treated 2	Treated 3	Median_control	Median_treated	Median_treated-Median_control
2	1	0.758663731	0.34820965	0.003876436	0.642442876	1.000717763	0.8555833	0.34820965	0.8555833	0.51
3	2	0.130918715	0.271232571	0.265793971	-0.141522901	0.109326558	-0.140060667	0.265793971	-0.140060667	-0.41
4	9	-0.128243647	0.410226506	0.324778604	-0.089392895	-0.305510407	-0.181695498	0.324778604	-0.181695498	-0.51
5	10	-0.077163476	0.123823371	-0.076933298	-0.186119858	-0.52990747	-0.45937185	-0.076933298	-0.45937185	-0.38
6	13	-0.510493578	0.087915244	-0.475235958	-0.514722273	-0.813358563	-0.705686289	-0.475235958	-0.705686289	-0.23
7	15	0.296868163	0.072493963	-0.129715961	0.347046645	0.577300684	0.272964787	0.072493963	0.347046645	0.27
8	16	-0.263026776	-0.353047701	-0.475362542	0.018696938	0.153800629	0.067445824	-0.353047701	0.067445824	0.42
9	18	-0.013300089	0.62666535	0.399959373	-0.14780107	-0.055482078	-0.163430139	0.399959373	-0.14780107	-0.55
10	19	0.16698505	-0.688921992	-0.50215357	0.152363706	0.117316609	0.083090417	-0.50215357	0.117316609	0.62
11	20	0.213366784	0.031479931	-0.113274138	0.214590824	0.363301003	0.358451706	0.031479931	0.358451706	0.33
12	25	0.214422514	-0.008519571	-0.111737977	0.207678953	0.251276396	0.302474176	-0.008519571	0.251276396	0.26
13	26	0.823363368	1.224624636	1.28616476	0.764994486	0.881133427	0.724969822	1.224624636	0.764994486	-0.46
14	27	0.346696071	0.165710926	-0.098466721	0.228977305	0.6624994	0.544969621	0.165710926	0.544969621	0.38
15	28	0.508291407	0.190267456	-0.004450423	0.5321771	0.657062276	0.693396938	0.190267456	0.657062276	0.47

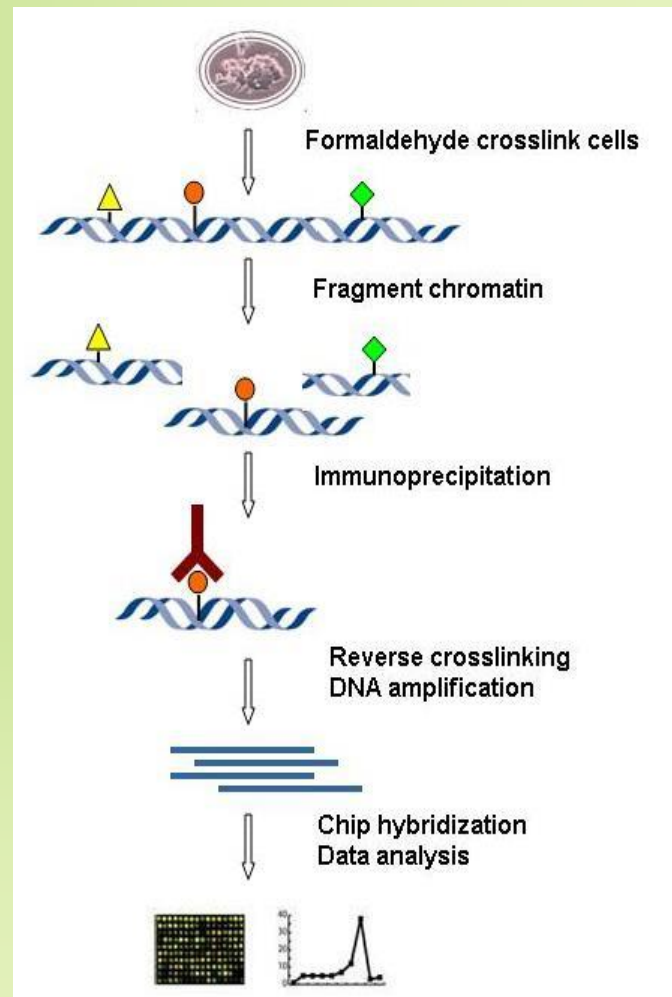
- List of NCBI gene identifiers with replicate data including median values for control and treated, and a final median log ratio for treated minus control to indicate hyper- (>0) or hypo- (<0) methylation.
- Information on location of methylation and annotation can be added (chromosome location; primary transcript, transcription start site).

# B. Histone Acetylation

## Overall procedure:

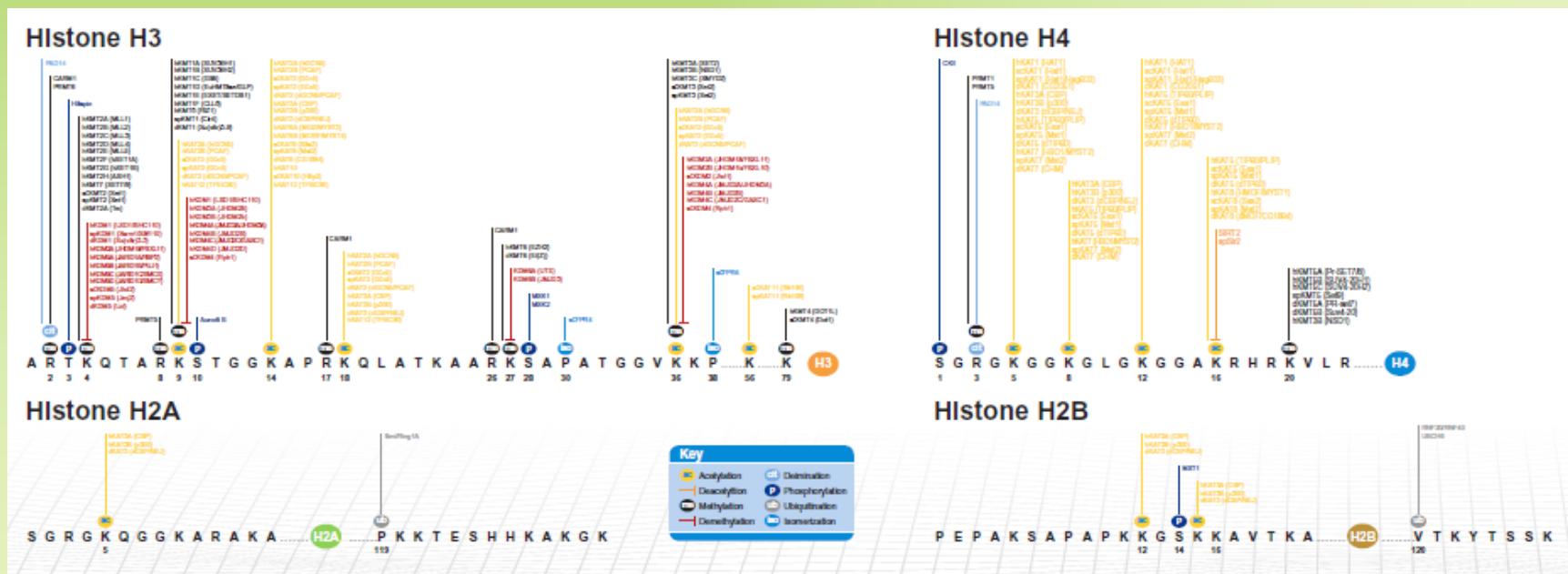
1. Cells are fixed with formaldehyde to cross-link histone and non-histone proteins to DNA
2. Cells are isolated and chromatin is digested with micrococcal nuclease and sonication (150-900 bp; minimal 3 bands)
3. Chromatin Immunoprecipitation using antibody against Acetyl-Histone H3 Lysine plus QC
4. Crosslinks are reversed
5. Microarray processing: labeling, hybridisation, washing, scanning  
(Roche NimbleGen)
6. Data extraction plus QC
7. Data analyses

As  
MeDIP-  
Chip



# B. Histone Acetylation

Cell signaling: SimpleChIP® Enzymatic Chromatin IP Kits  
Start amount cells: 5\*10E6  
Antibody: Acetyl-Histone H3 (Lys9) (C5B11) Rabbit mAb #9649  
→ H3K9Ac: classic histone mark: activation  
(Berger SL. Nature 2007; 447:407-12)



# C. miRNA

## Overall procedure:

1. Cell lysis using Qiazol
2. miRNA isolation using miRNeasy minikit
3. QC (integrity: Bioanalyzer) plus nanodrop measurements (yield, purity)
4. Microarray processing: labeling, hybridisation, washing, scanning (Agilent Technologies)
5. Data extraction plus QC
6. Data analyses

# C. miRNA

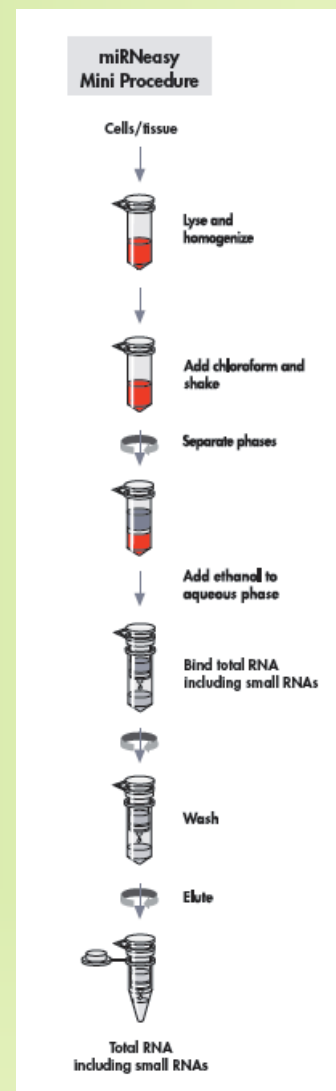
## Overall procedure:

1. Cell lysis using Qiazol
2. miRNA isolation using miRNeasy minikit
3. QC (integrity: Bioanalyzer) plus nanodrop measurements (yield, purity)

RIN > 8

18S and 28S peak; no degradation

Purity A260/A280 : > 1.9





# C. miRNA

## Overall procedure:

### 4. Microarray processing: labeling, hybridisation, washing, scanning (Agilent Technologies)

Total RNA (100 ng) + Labeling Spike-In (optional)

↓ Phosphatase Treatment, incubate 30 minutes, 37°C\*

Dephosphorylated RNA

↓ Add DMSO

↓ Heat, ice

↓ Assemble Labeling Reaction, incubate 2 hours, 16°C\*

Labeled RNA

↓ Desalt with Spin Column (optional)\*

Desalted Labeled RNA

↓ Dry sample with vacuum concentrator, approximately 2 to 3 hours (1 hour with columns), 45°C to 55°C\*

↓ Assemble Hybridization Mixture + Hyb Spike-In (optional)

↓ Heat, ice

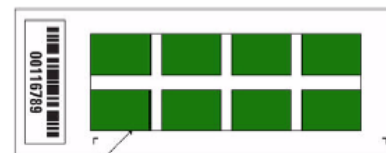
↓ Hybridize 20 hours, 55°C, 20 RPM

↓ Wash, Scan

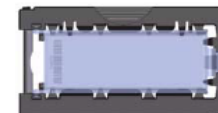
miRNA Profile

Agilent oligo microarray (8 microarray/slide format) as imaged on Agilent microarray scanners

Microarrays are printed on the side of the glass labeled with the "Agilent" bar code (also called the "active side" or "front side").



Agilent Microarray Scanner scans through the glass. (Back side scanning.)



Agilent microarray slide holder for SureScan (above) and Scanner B/C (below) microarray scanners.



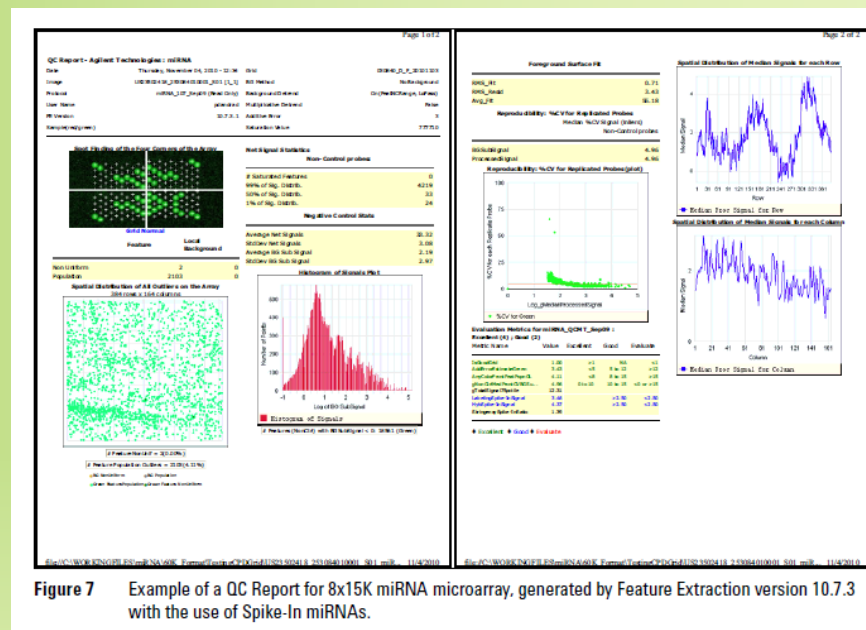
# C. miRNA

## Overall procedure:

5. Data extraction plus QC

6. Data analyses

- Bioconductor R-script (AgiMicroRna package)
- Total microRNA Gene Signal processed by Agilent Feature extraction algorithm



**Figure 7** Example of a QC Report for 8x15K miRNA microarray, generated by Feature Extraction version 10.7.3 with the use of Spike-In miRNAs.

# C. miRNA

## 6. Data analyses continued

- Normalization of the arrays;
- Linear model features implemented in Limma;
- List of differentially expressed microRNAs

Two databases are currently used for target gene identification:

→ Validated targets:

MiRTtarBase Release 4.5: Nov. 1, 2013.

(<http://mirtarbase.mbc.nctu.edu.tw/>)

→ Predicted targets:

TargetScan Release 6.2: June, 2012.

([http://www.targetscan.org/vert\\_61/](http://www.targetscan.org/vert_61/))

# C. miRNA: example results file

## Differentially expressed miRNAs

M: log-ratio

A: mean average  
expression

fdr.pval: FDR

adjusted p-value

	A	B	C	D	E	F	G	H
1	PROBE	GENE	M	A	t	pval	adj.pval	fdr.pval
2	A_25_P00012870	hsa-miR-454	-4.277	1.139	-26.261	0	0.00021	0.00021
3	A_25_P00010616	hsa-miR-137	-4.25	1.125	-19.969	0	0.00044	0.00044
4	A_25_P00013752	ebv-miR-BART12	-5.137	1.568	-17.921	1.00E-05	0.00052	0.00052
5	A_25_P00015648	hsa-miR-4281	1.497	9.211	12.544	3.00E-05	0.00231	0.00231
6	A_25_P00010386	hsa-miR-146b-5p	-3.065	0.533	-12.266	4.00E-05	0.00231	0.00231
7	A_25_P00013252	hsa-miR-29b-1*	-2.922	0.461	-11.513	5.00E-05	0.00267	0.00267
8	A_25_P00012858	hsa-miR-542-5p	-3.085	0.543	-10.609	8.00E-05	0.0035	0.0035
9	A_25_P00012257	hsa-miR-193a-3p	-0.853	3.989	-9.96	0.00012	0.00424	0.00424
10	A_25_P00015912	hsv1-miR-H18	0.931	5.443	9.085	0.00018	0.00557	0.00557

## Differentially expressed miRNAs

Gene targets

	A	B	C
1	hsa-mir-454	FERMT2	10979
2	hsa-mir-454	CLOCK	9575
3	hsa-mir-454	ST8SIA5	29906
4	hsa-mir-454	SLMAP	7871
5	hsa-mir-454	PHAX	51808
6	hsa-mir-454	KLHDC8A	55220
7	hsa-mir-454	MAP4	4134
8	hsa-mir-454	FIBIN	387758
9	hsa-mir-454	NR3C2	4306
10	hsa-mir-454	HECA	51696

# (Integrative) Data Analyses

## A. Within one endpoint:

### **1. Interesting genes per time point**

- Based on a-priori knowledge
- Targeted approach:  
→ *Text mining Databases: e.g. Comparative Toxicogenomics database*

Which genes are known to play a role in the particular disease?

Which genes are modulated by the investigated compound?

Which genes are involved in compound induced disease?

→ Which of these genes are present in the datasets?

- Pathway analyses using significant gene lists

# (Integrative) Data Analyses

## B. Between different -omics analyses

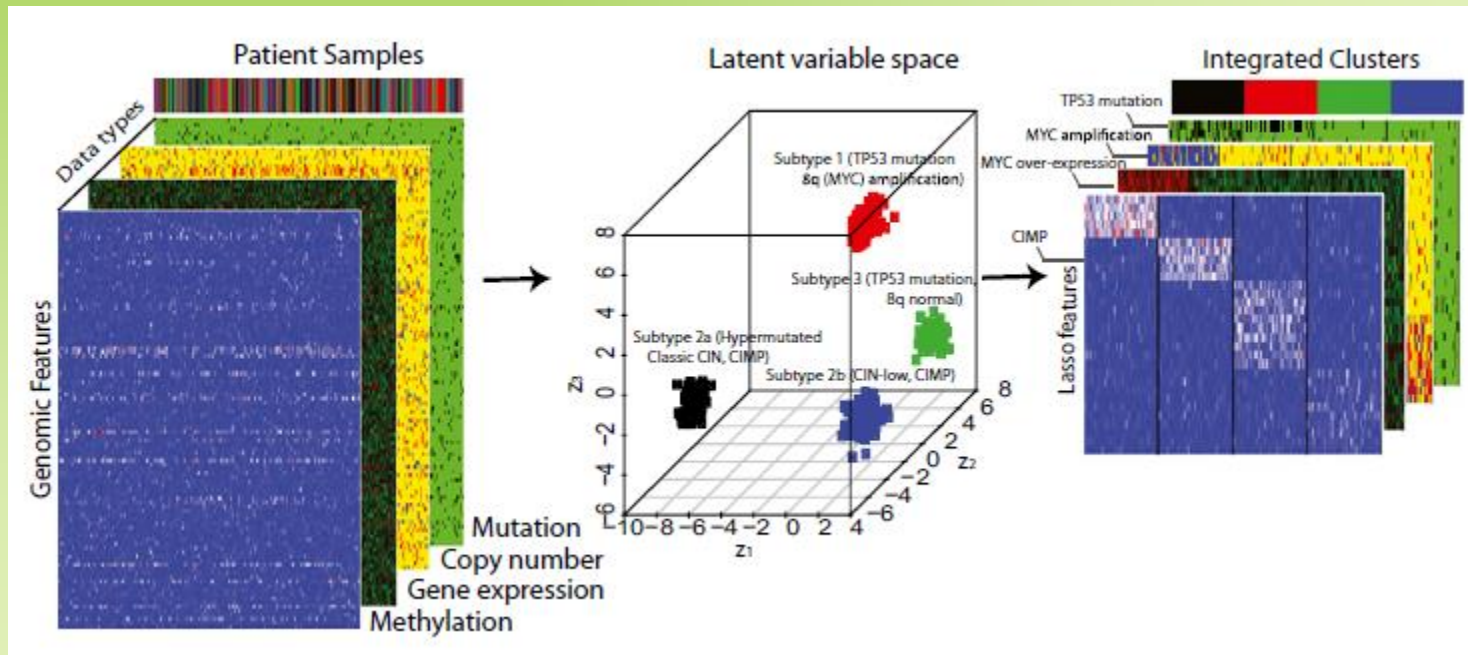
- Which differentially expressed genes show anti-correlation with its DNA methylation change?
- Which differentially expressed genes show correlation with its histone acetylation change?
- Which DEGs show correlation with its DNA methylation change? For which of these genes miRNA expression data are available?
- Are target genes of differentially expressed miRNAs modulated?

→ *Pathway analyses of overlapping gene list*



# Integrative Data Analyses: iCluster+

- Developed for predicting cancer subtypes
- Can also be used for clustering of compounds
- Integrates discrete and continuous variables
- Clusters patients, compounds, samples
- Provides a list of top features within each data type (based on lasso coefficient estimates)



<http://www.mskcc.org/research/epidemiology-biostatistics/biostatistics/iclusterplus>

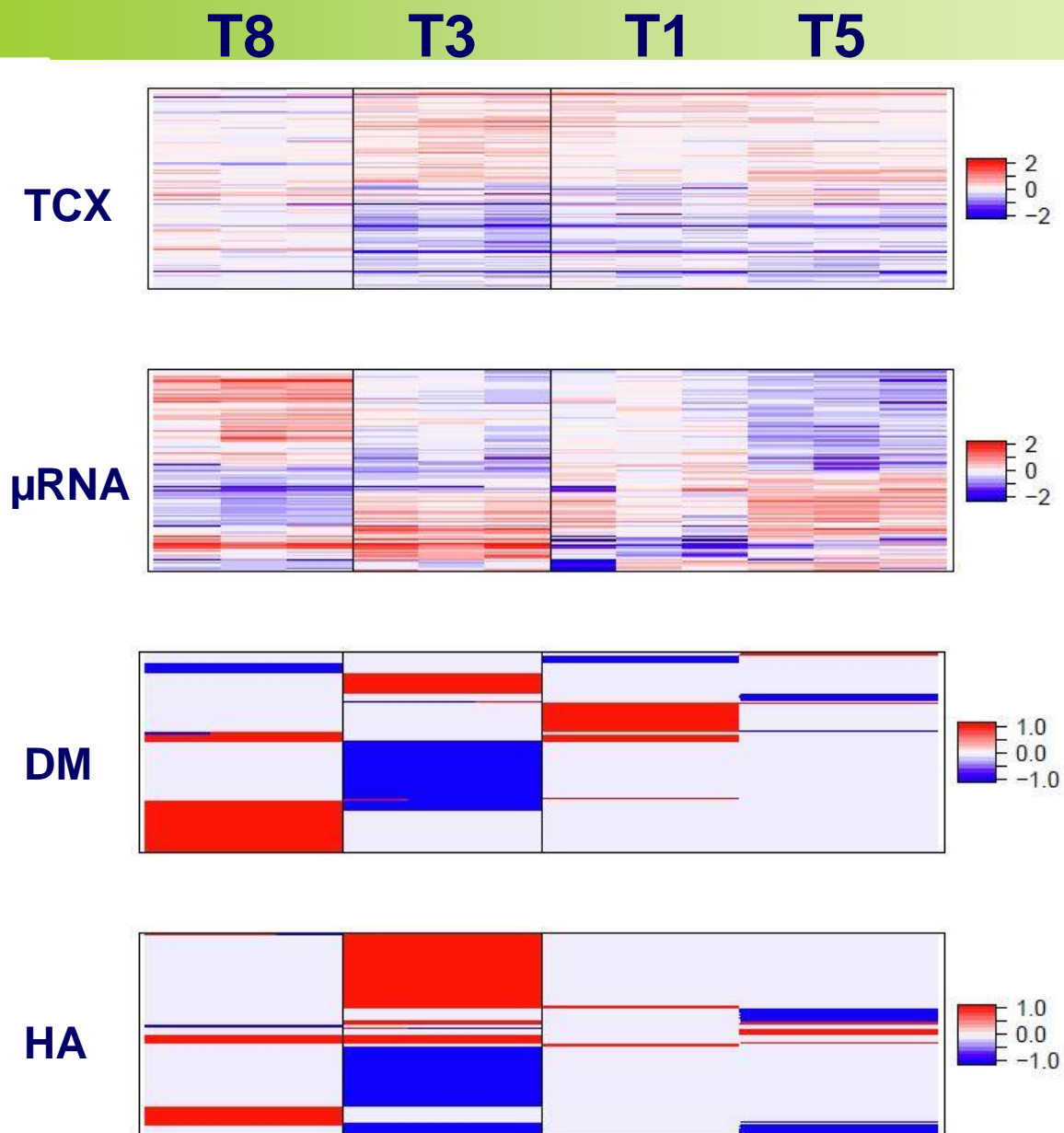
R package: iClusterPlus

Shen et al. 2009

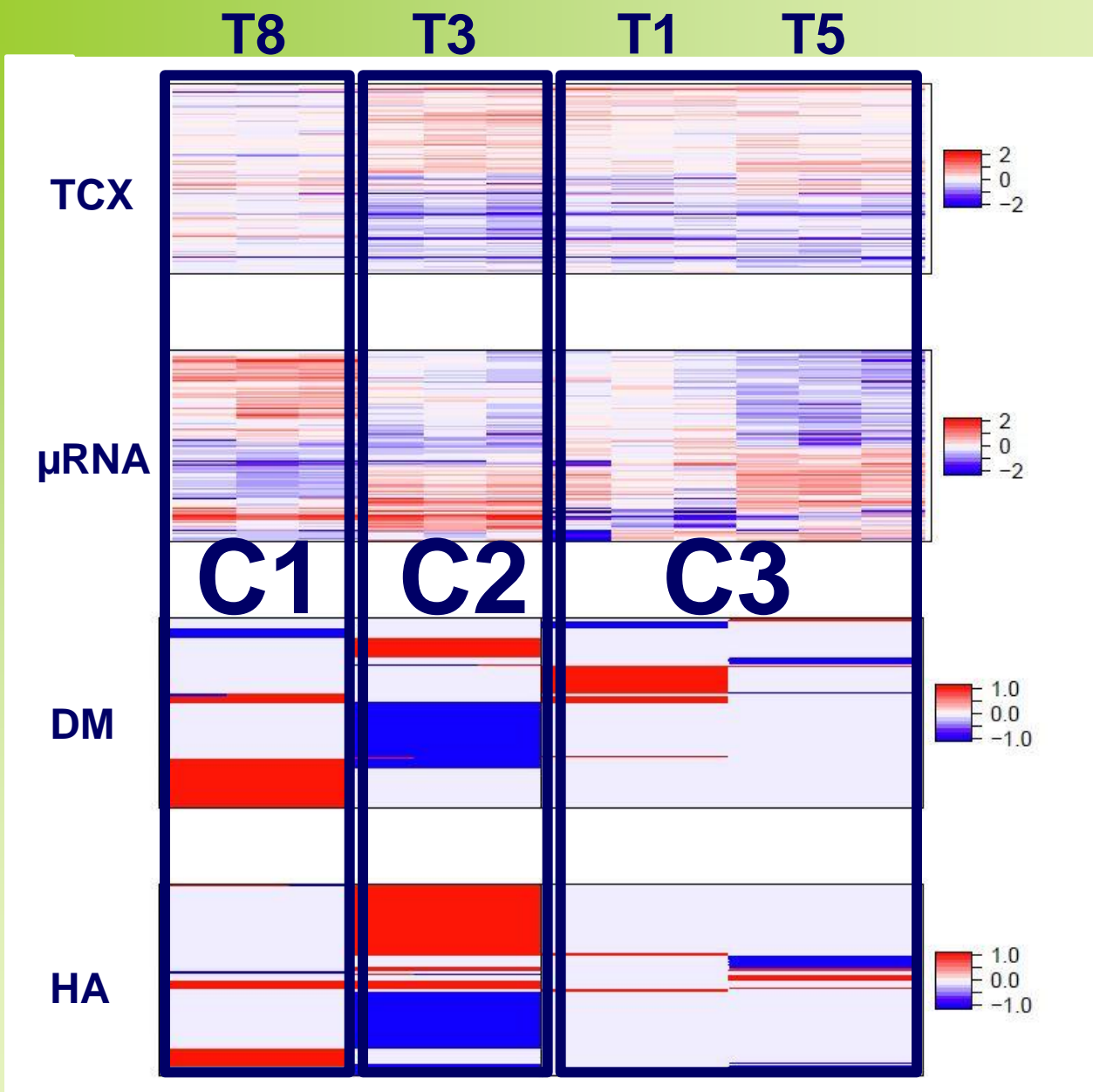
## Selection criteria for used datasets:

- 1) Transcriptomics:
  - all genes were selected that were significant ( $P < 0.05$ ) for at least one experimental condition;
  - Only probesets for which an EntrezGene ID were present were included
  - Probesets with the same EntrezGene ID were merged (median)
- 2) miRNA expression: all genes were selected that were significant ( $P < 0.05$ ) for at least one experimental condition
- 3) DNA methylation: genes were selected which were significantly ( $P < 0.01$ ) hyper- or hypo-methylated in at least one experimental condition
- 4) Histone acetylation: genes were selected which were significantly ( $P < 0.01$ ) hyper- or hypo-acetylated in at least one experimental condition

# iCluster+



# iCluster+



# 3. Conclusions

## Epigenetics:

- Gene expression regulation without changing the DNA sequence
  - you are more than the sum of your genes
- DNA methylation, histone modification, miRNA
- Epigenomics analyses at Maastricht University
- (integrative) data analyses challenging!

