

DNA Methylation in Multiple Myeloma

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- What is DNA methylation?
 - Epigenetics
 - Other linked mechanisms
 - What does it do?
 - Why is it important generally?
 - Why is it important in cancer biology?

- How is it measured?
- What has been done in myeloma?

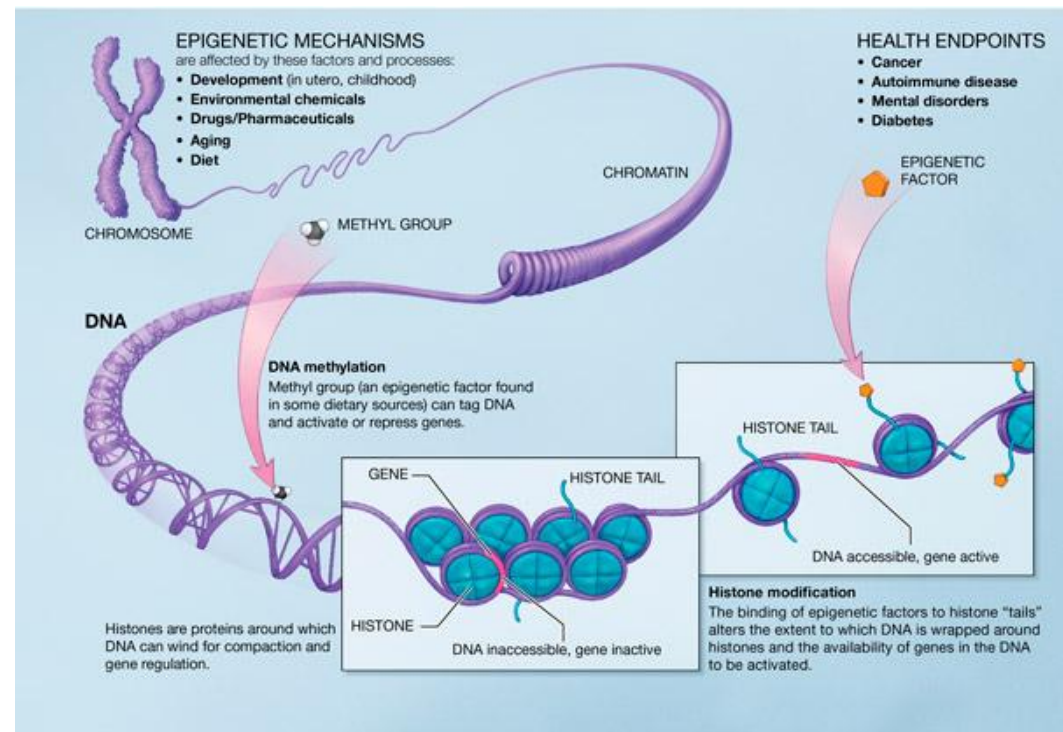
- Our own work with DNA methylation arrays
 - investigation of plasma cell types
 - integration with previous gene expression and cytogenetic datasets

What is Epigenetics?

epigenetics is the study of heritable changes in phenotype or gene expression caused by mechanisms other than changes in the underlying DNA sequence, hence the name *epi-* (Greek: *επί-* over, above) - *genetics*

Changes in gene expression not caused by mutation, deletion, amplification or translocation

1. DNA methylation
2. Histone modifications

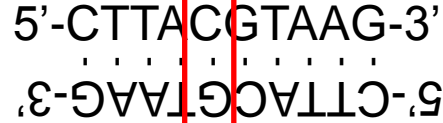
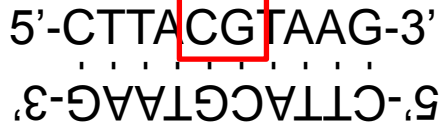


1. DNA Methylation

DNA is composed of four bases – A, G, C, T

The 'fifth base' is known as methyl-C

- methyl-C is only found in a CpG dinucleotide



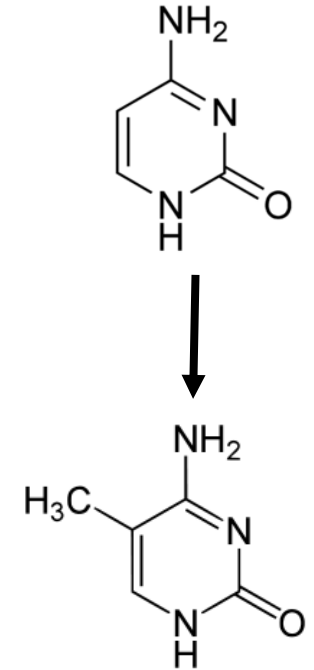
CpG dinucleotides are under-represented in the human genome

- expect 4.4% in a 42% GC rich genome but only find ~1%
- not uniformly distributed throughout the genome
- found in clusters called 'CpG islands'
- CpG islands are found at genic regions (7% mostly unmethylated), or in repetitive sequences (41% mostly methylated)

Controlled by DNA methyltransferases (DNMTs)

DNMT1 - maintains DNA methylation

DNMT3a/b - *de novo* DNA methylation



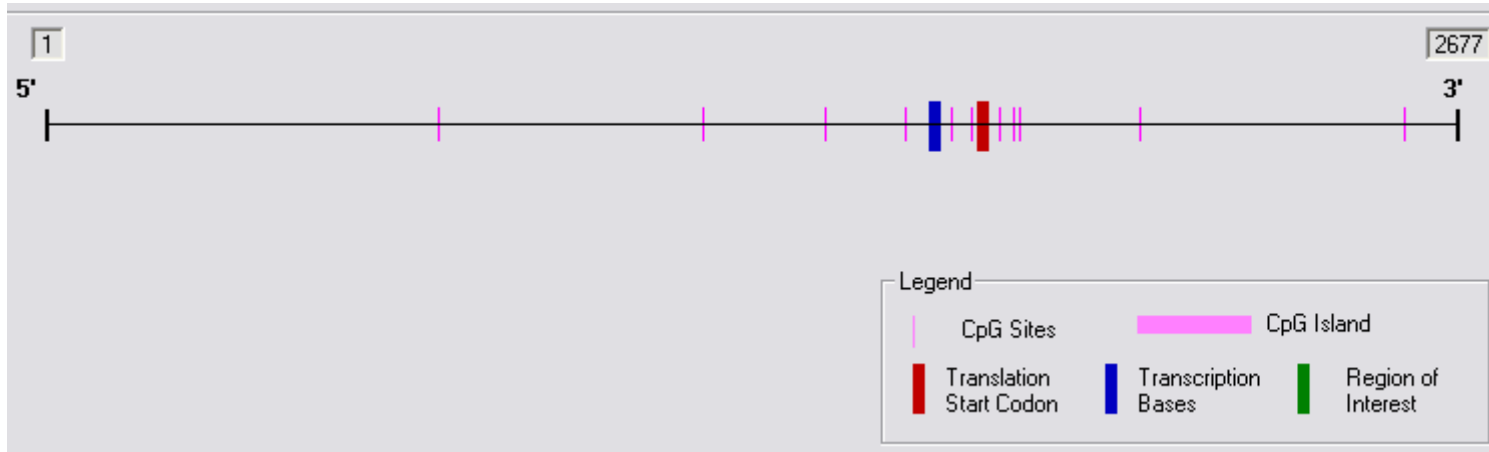
CpG Islands Are Found Surrounding The Start of Genes

CpG islands have a loose definition:

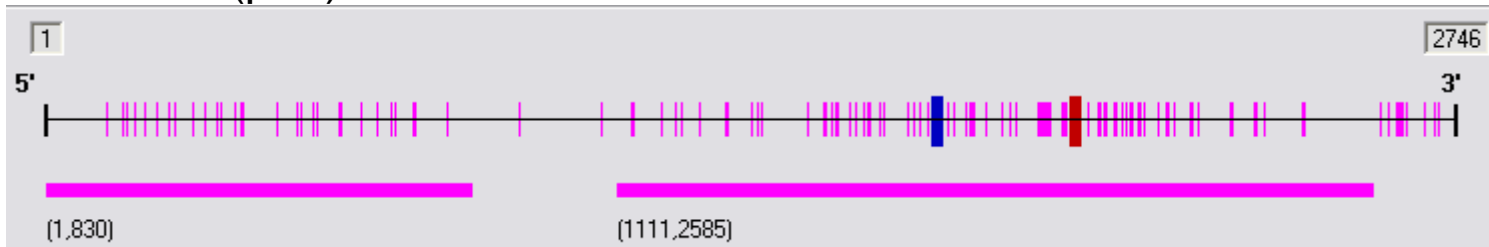
50% C/G

CpG Obs/Exp >0.6

Albumin



CDKN2A (p16)



2. Histone Modifications

DNA is wrapped around histone proteins – complex termed chromatin

Histone tails can be modified through:

- methylation
- acetylation
- phosphorylation
- ubiquitylation

All these changes have a substantial influence on the chromatin structure and gene function and this influence is dependent on the type and location of the modification

For example:

H3K9-me and H3K27-me are associated with inactive transcription

H3K9-ac is associated with active transcription

Controlled by many proteins:

HMTs – histone methyltransferases e.g. MMSET

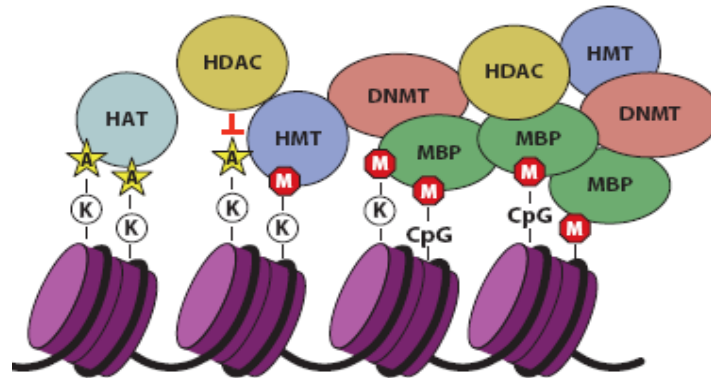
HAT – histone acetyltransferases

HDAC – histone deacetylases

Histone Demethylases e.g. UTX

Interplay of the Two Mechanisms

- DNA methylation is intrinsically linked to chromatin remodelling
 - Euchromatin (active) has an open structure, lack of DNA methylation and specific histone modifications (e.g. acetylated H3K9)
 - Active transcription
 - Heterochromatin (inactive) has a closed structure and is associated with DNA methylation and specific histone modifications (e.g. methylated H3K9 and H4K20)
 - Inactive transcription
- The state is controlled by many proteins that interact with both the histones and CpG dinucleotides in the DNA



- HMTs can recruit DNMTs resulting in increased methylation of histones and DNA

Methylation and Biology

- DNA methylation is important in normal cellular function

In development:

- methylation changes allow cells to develop into different cell types, or revert to stem cells

Imprinting:

- whereby alleles inherited from paternal or maternal source have different methylation patterns
 - Angelman Syndrome, Prader-Wili Syndrome
 - result of loss of expression of the relevant gene

Methylation in Cancer

Evidence in many cancer types of abnormal methylation patterns compared to normal tissues

- 1- Wave of loss of DNA methylation (hypo-methylation) across the genome
 - results in genome instability, may lead to chromosomal alterations such as amplifications, deletions or translocations
 - mostly found in repetitive elements

- 2- Gene-specific hyper-methylation
 - increased methylation at tumour suppressor genes
 - associated with loss in expression
 - mostly found in genic regions

Initiating factors not known

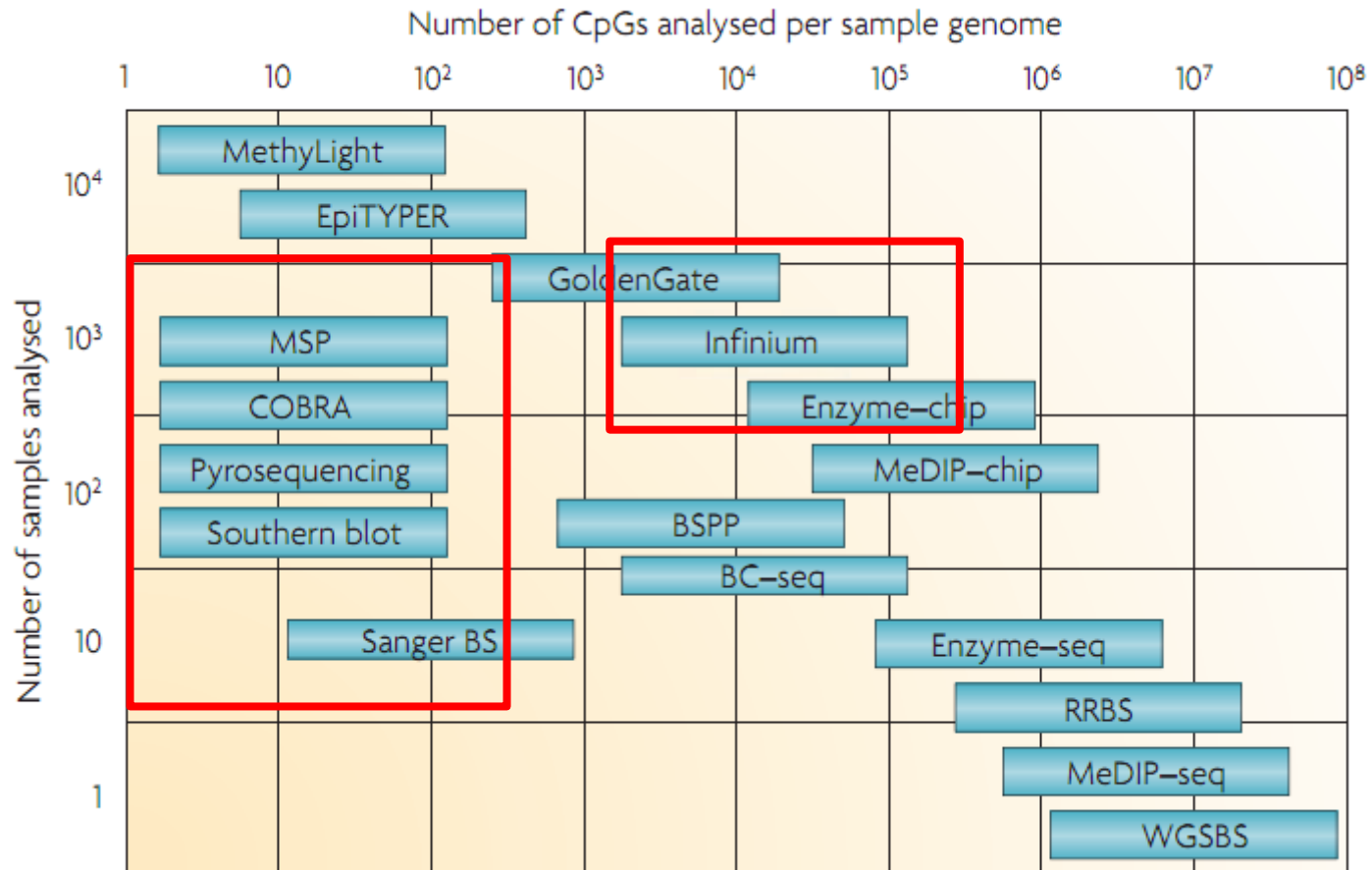
Changes in methylation can alter TSGs or oncogenes

increase in methylation of TSG

decrease in methylation of oncogene

methylation of other regulatory elements – transcription factors, miRNAs etc.

Approaches to Analysing the Methylome



Methylation in Myeloma

- Methylation in myeloma has generally been studied on a gene-by-gene basis
- Identified hypermethylation of specific genes e.g. *CDH1* (E-cadherin), *SOCS1*, *CDKN2A*, *CDKN2B*, *TGFBR2* etc.
- Hypermethylation of these genes are associated with poor outcome
- Use of demethylating agents in treating patients suggests that the methylome is important in myeloma
- Whole methylome analysis can be used to identify new targets and mechanisms of disease progression

Differential repetitive DNA methylation in multiple myeloma molecular subgroups

Valentina Bollati, Sonia Fabris¹, Valeria Pegoraro,
Domenica Ronchetti¹, Laura Mosca¹, Giorgio
Lambertenghi Delilieri¹, Valeria Motta, Pier Alberto
Bertazzi, Andrea Baccarelli and Antonino Neri^{1,*}

genetic instability are poorly understood. Epigenetics relate to stable and heritable patterns of gene expression and genomic functions that do not involve changes in DNA sequence (6). In mammals, DNA methylation, the most investigated epigenetic hallmark, is a reversible mechanism that modifies genome function and chromosomal stability

Tumor and Stem Cell Biology

Cancer
Research

DNA Methylation Analysis Determines the High Frequency of Genic Hypomethylation and Low Frequency of Hypermethylation Events in Plasma Cell Tumors

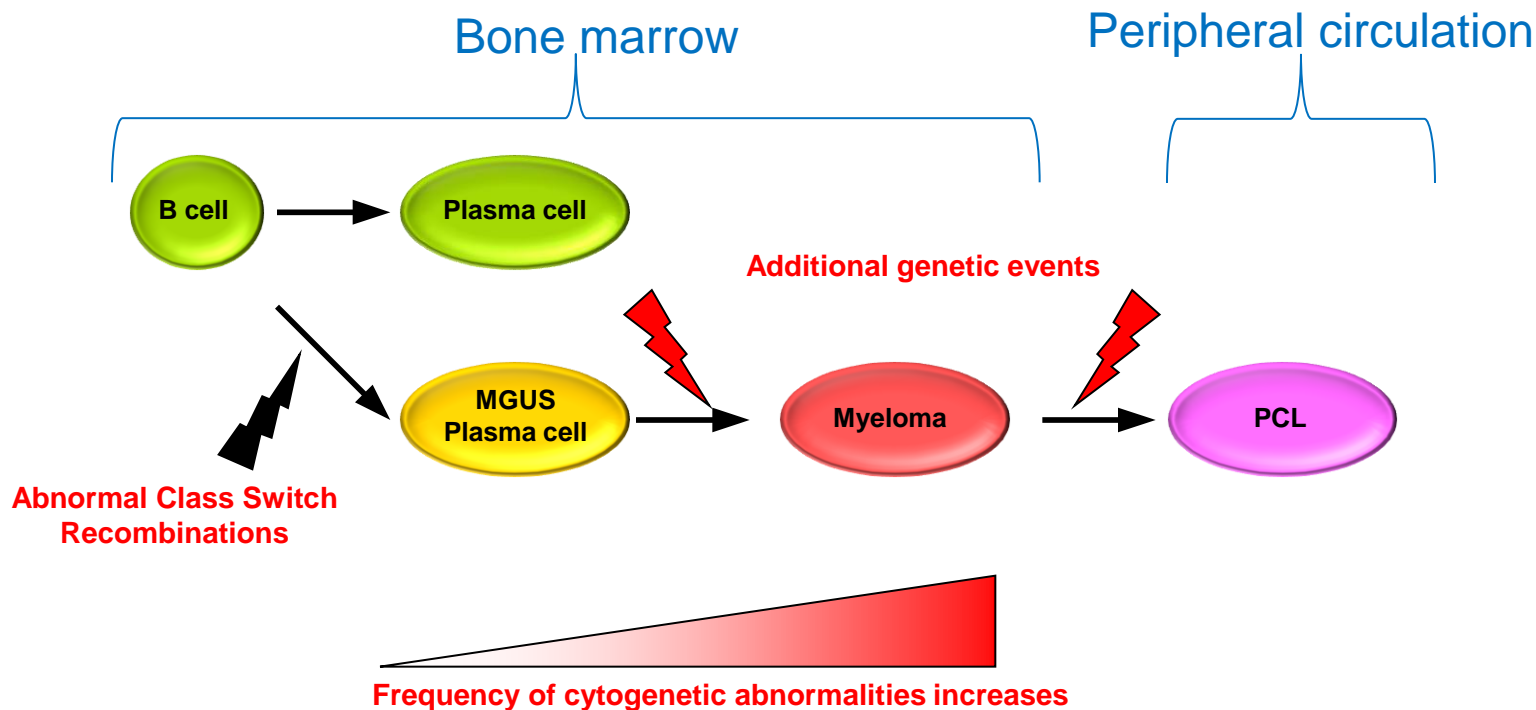
Bodour Salthia¹, Angela Baker¹, Gregory Ahmann², Daniel Auclair³, Rafael Fonseca², and John Carpten¹

Abstract

Multiple myeloma (MM) is a plasma cell malignancy of the bone marrow, which evolves from a premalignant stage called monoclonal gammopathy of undetermined significance (MGUS). In some patients, an intermediate stage referred to as smoldering multiple myeloma (SMM) is clinically recognized, with the full-blown malignancy termed MM. We conducted a study to assess differential CpG methylation at 1,500 genic loci during MM progression and profiled CD138⁺ plasma cells from MGUS, SMM, and MM specimens; human myeloma cell lines; and normal plasma cell (NPC) samples. We showed that the number of differentially methylated loci (DML) increased with tumor grade, and the vast majority were due to hypomethylation. Hierarchical clustering analysis revealed samples that coclustered tightly with NPC. These cases, referred to as “normal-like,” contained significantly fewer DML when compared with their non-normal-like counterparts and displayed overall methylation levels resembling NPC. This study represents one of the first methylome interrogation studies in MM and points toward global hypomethylation at genic CpG loci as an important and early mechanism driving myelomagenesis. Determining the set of critical genes and pathways based on the myeloma methylome is expected to lead to an improved understanding of biological mechanisms involved in myelomagenesis. *Cancer Res*; 70(17): 6934–44. ©2010 AACR.

Myeloma Biology

- Myeloma occurs during the normal maturation of B cells into Plasma cells
- Generate a non-malignant state known as MGUS
- Further events transform the cell into a malignant clone causing disease symptoms
- Late stage disease can result in bone marrow independence

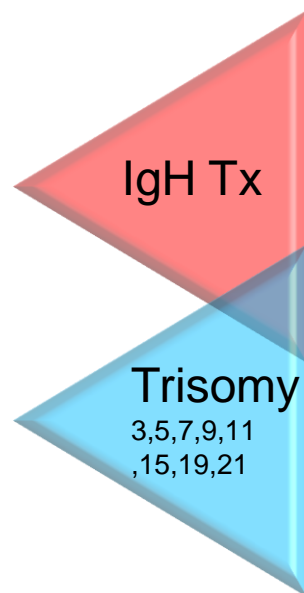


Plasma cell involvement:	MGUS <10%	Myeloma >10%
Lytic bone lesions:	None	Many
Progression to MM pa:	1%	-
Median years to progression:	>25	-

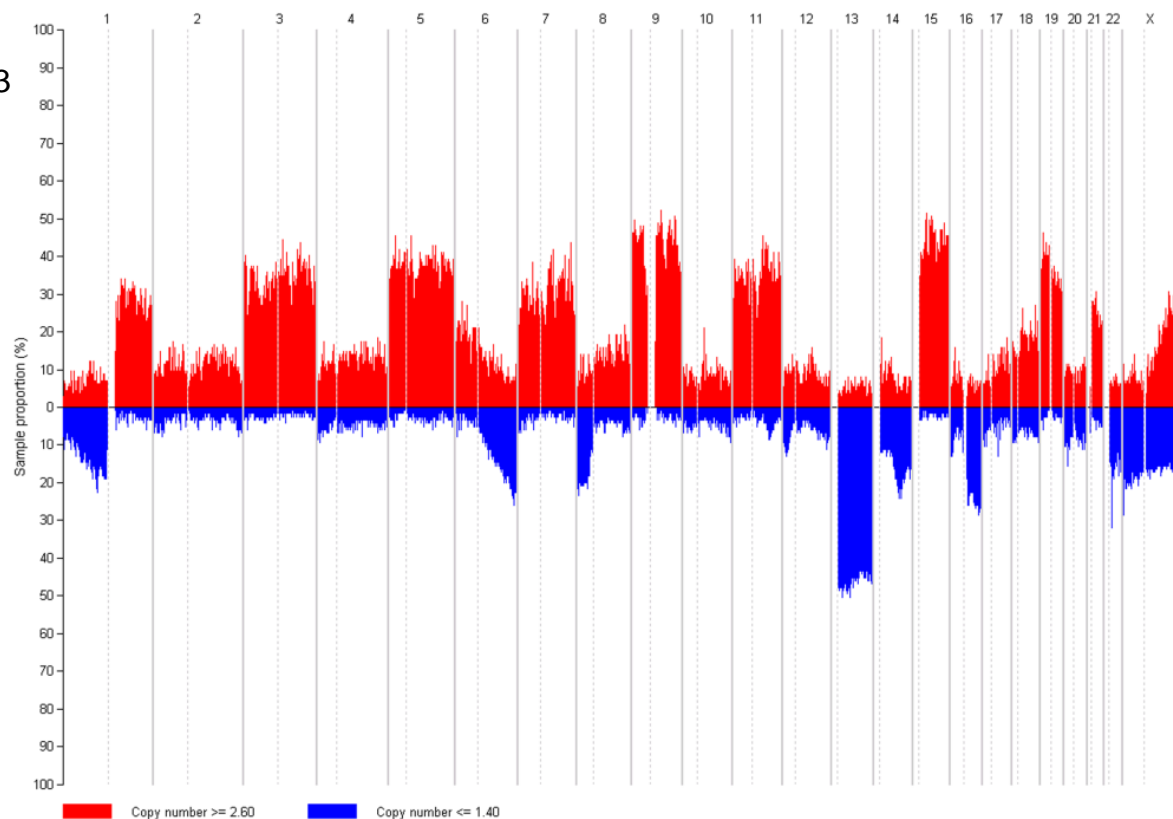
Does DNA methylation play a part in this process?

Myeloma Genetics

- Myeloma genomes characterised by primary events: IGH Translocations, hyperdiploidy
- Copy number abnormalities: gain 1q, deletion of 1p, 6q, 8p, 13q, 16q, 17p

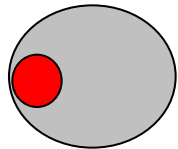


4p16 – MMSET/FGFR3
6p21 – CCND3
11q13 – CCND1
16q23 – MAF
20q11 - MAFB



Myeloma IX Samples

Bone marrow-derived
CD138+

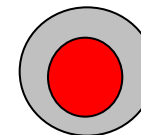


Lysis

AllPrep spin
columns
Simultaneous
extraction of RNA
and DNA

CELLS
FISH and
cytogenetics
>1700

PBL



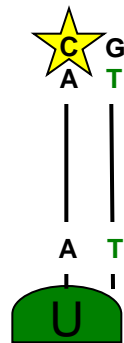
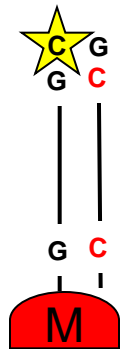
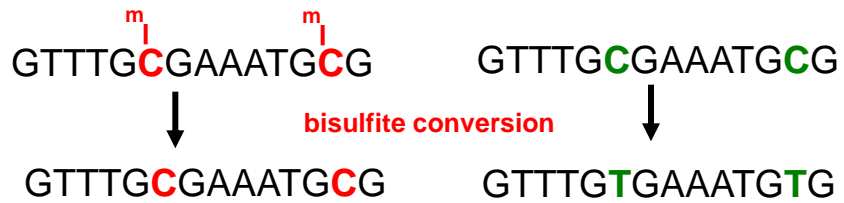
RNA
Gene Expression
U133 Plus 2.0
258 samples

DNA
Genomic Analysis
500K Mapping Set
115 samples

DNA
Genomic Analysis

CLINICAL DATA
OS, PFS, etc.

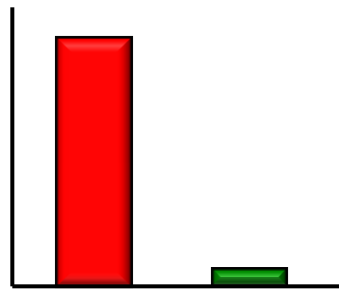
Illumina Infinium Global Methylation Array



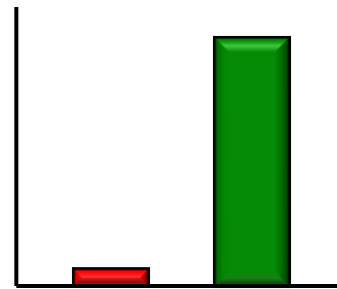
- Humanmethylation27 beadarray
- 27,578 informative CpG sites
- 500 ng DNA

$$\frac{M}{M+U} = \text{proportion methylated}$$

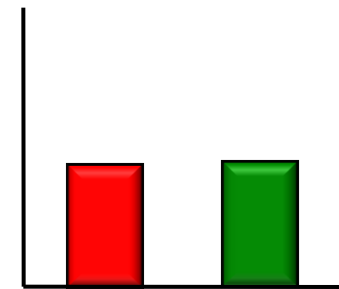
1.0 fully methylated
0.0 fully unmethylated



Methylated

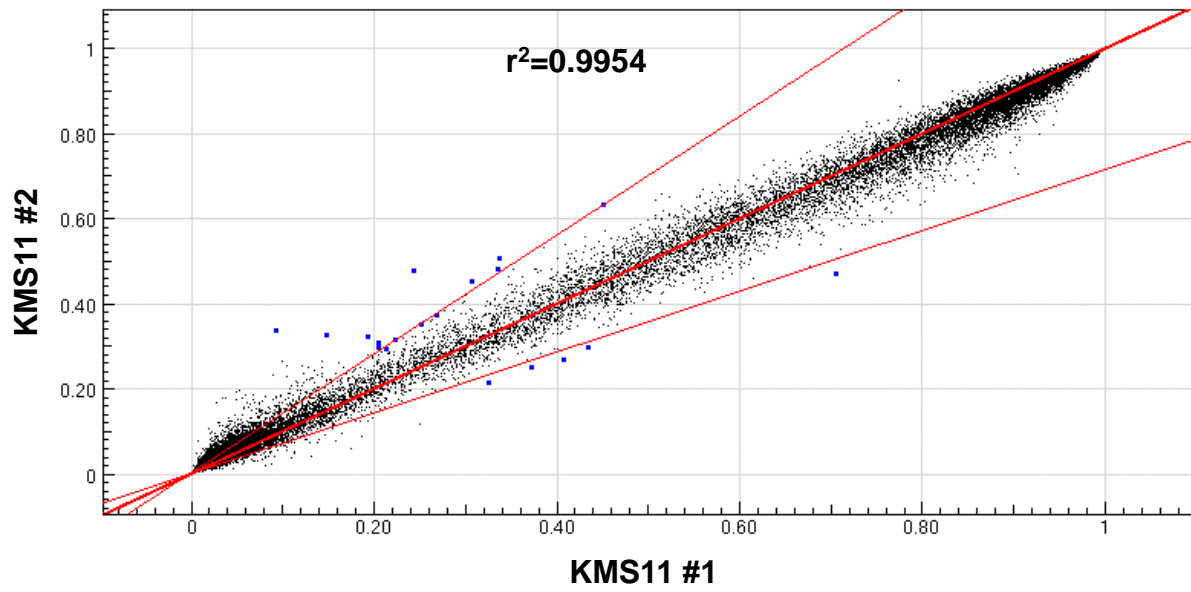


Unmethylated

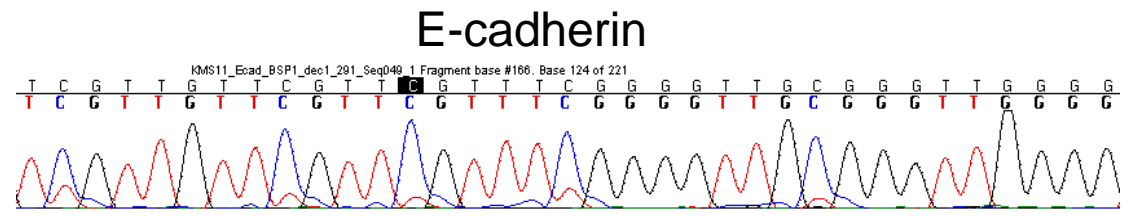
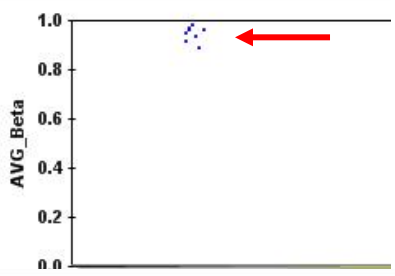


Hemi-methylated

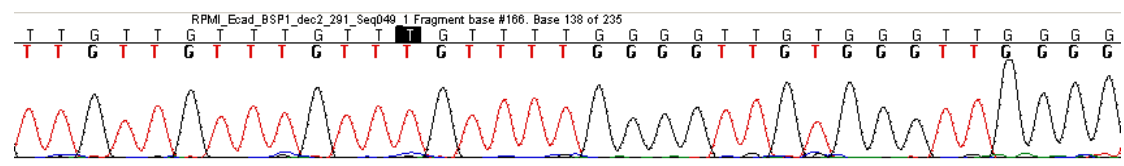
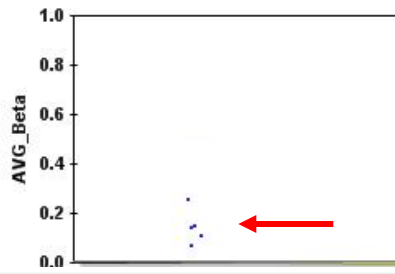
Validation of methylation by BSP sequencing



KMS11



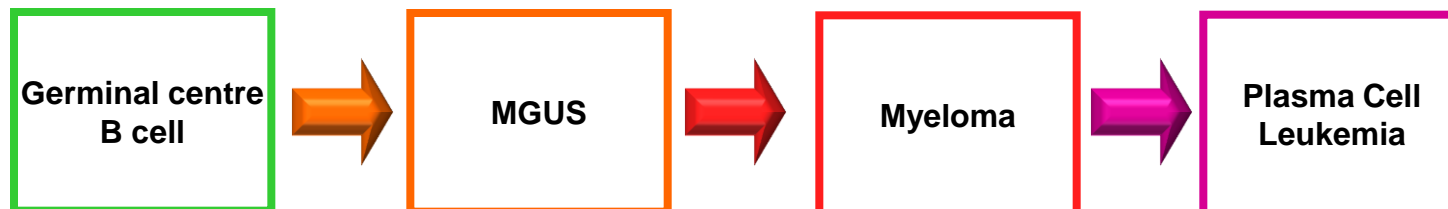
RPMI-8226



Samples

Type	Expression	Methylation
B cell		6
Normal PC		4
MGUS		5
Myeloma	205	161
Plasma Cell Leukemia	10	32
HMCL		6

	%		%
t(4;14)	9	t(4;14)	16
t(6;14)	1	t(6;14)	0
t(11;14)	21	t(11;14)	28
t(14;16)	5	t(14;16)	31
t(14;20)	2	t(14;20)	3
HRD	42	HRD	9
Del(1p32.2)	13	Del(1p32.2)	29
Del(13q)	43	Del(13q)	57
Del(16q)	24	Del(16q)	29
Del(17p)	5	Del(17p)	34
Gain 1q	29	Gain 1q	60



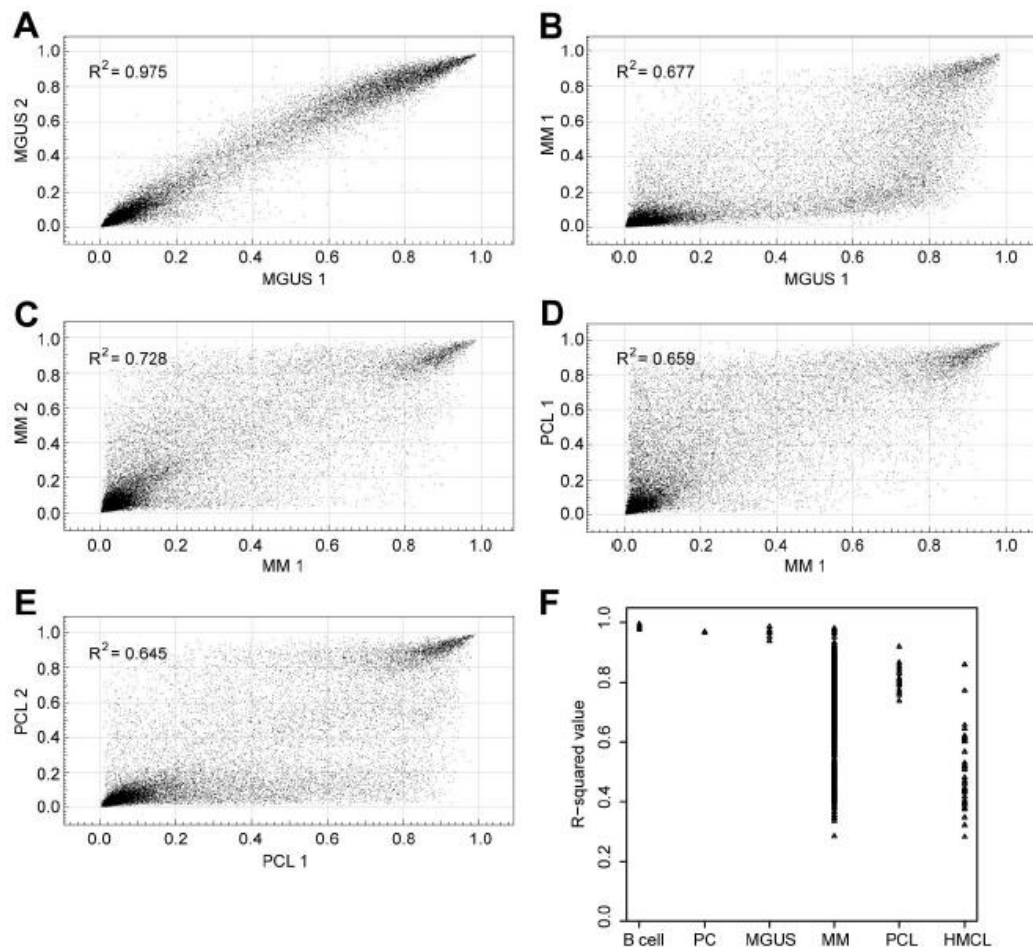
Overall Methylation Differs Between Cell Types

- Little variation in methylation between MGUS samples (A)

- Large variation between MGUS and MM samples (B) and between MM samples (C)

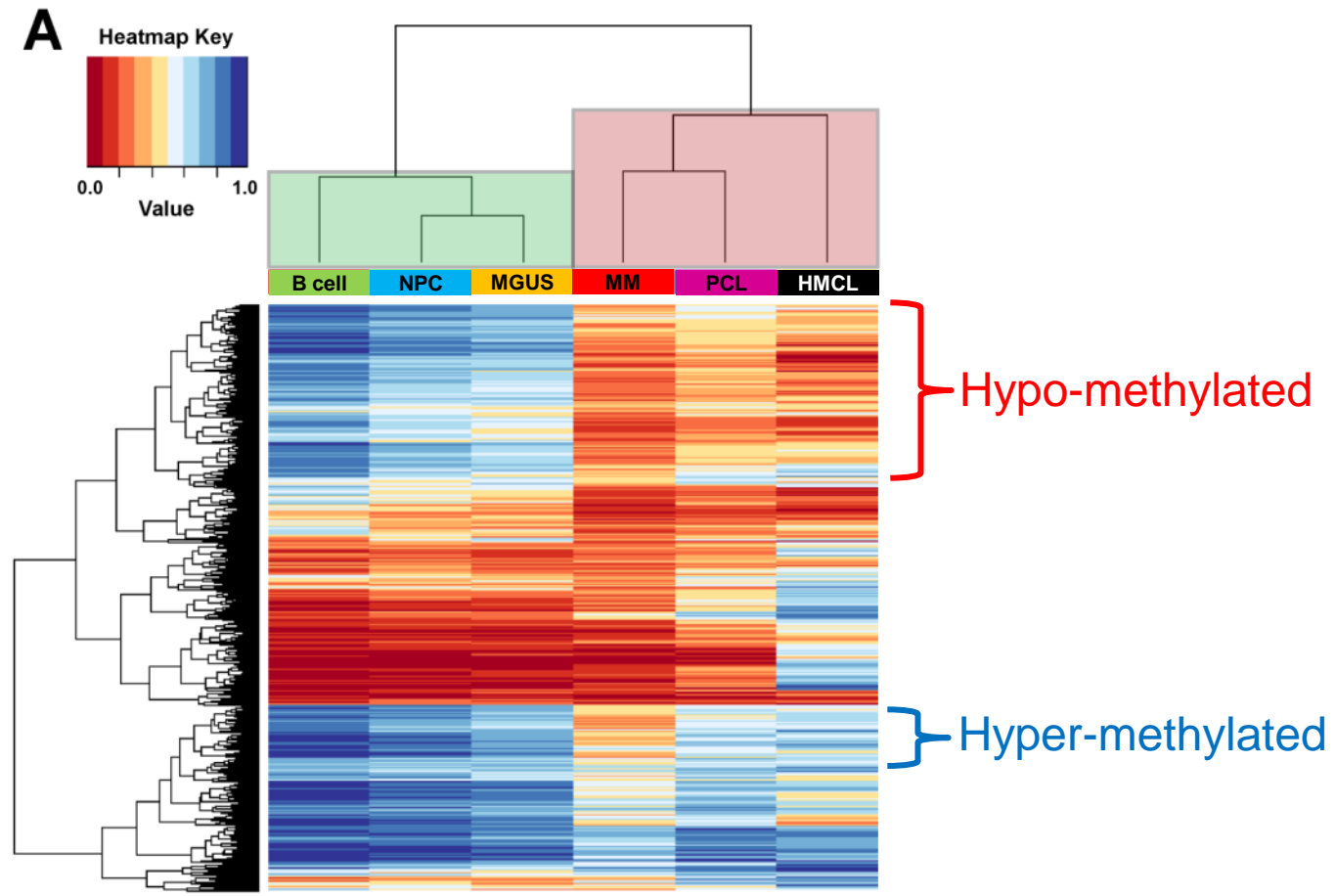
- Large variation between MM and PCL samples (D) and between PCL samples (E)

- Malignant phenotypes are more heterogeneous than non-malignant phenotypes (F)



Methylation status defines myeloma progression

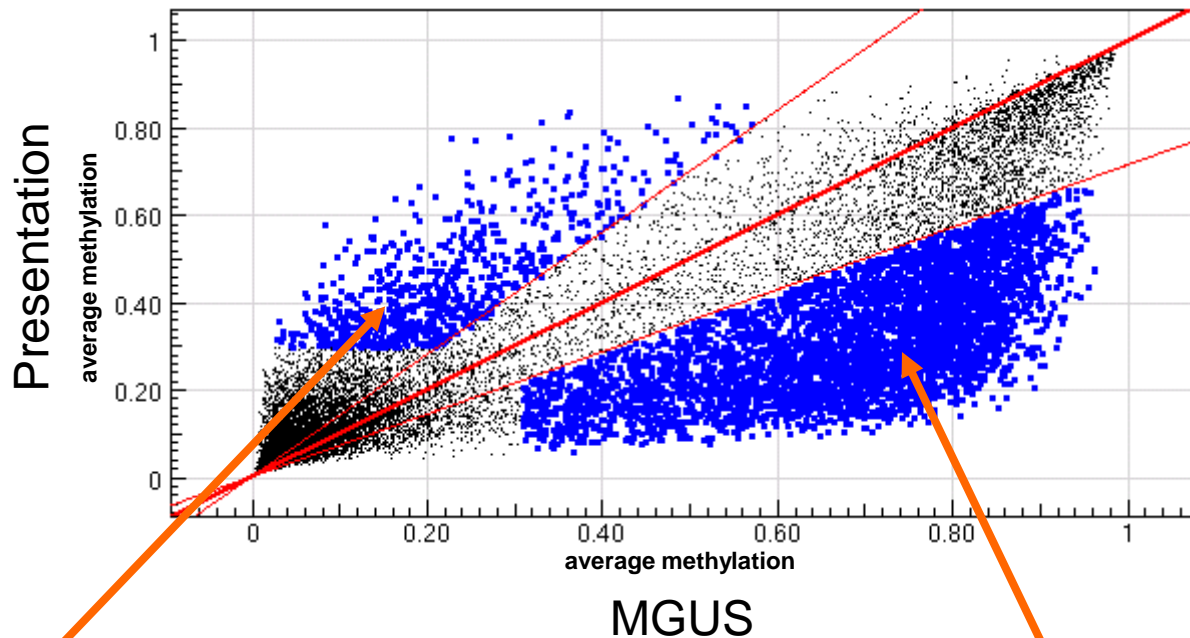
Supervised clustering based on sample cell type



B

	B cell	PC	MGUS	MM	PCL	HMCL
Hypo	637	14	4620	425	41	
Hyper	222	9	784	1148	426	

Methylation changes in disease progression



MGUS → MM Hyper-methylation

784 probes (2.8%)

675 unique genes (5.5%)

MGUS → MM Hypo-methylation

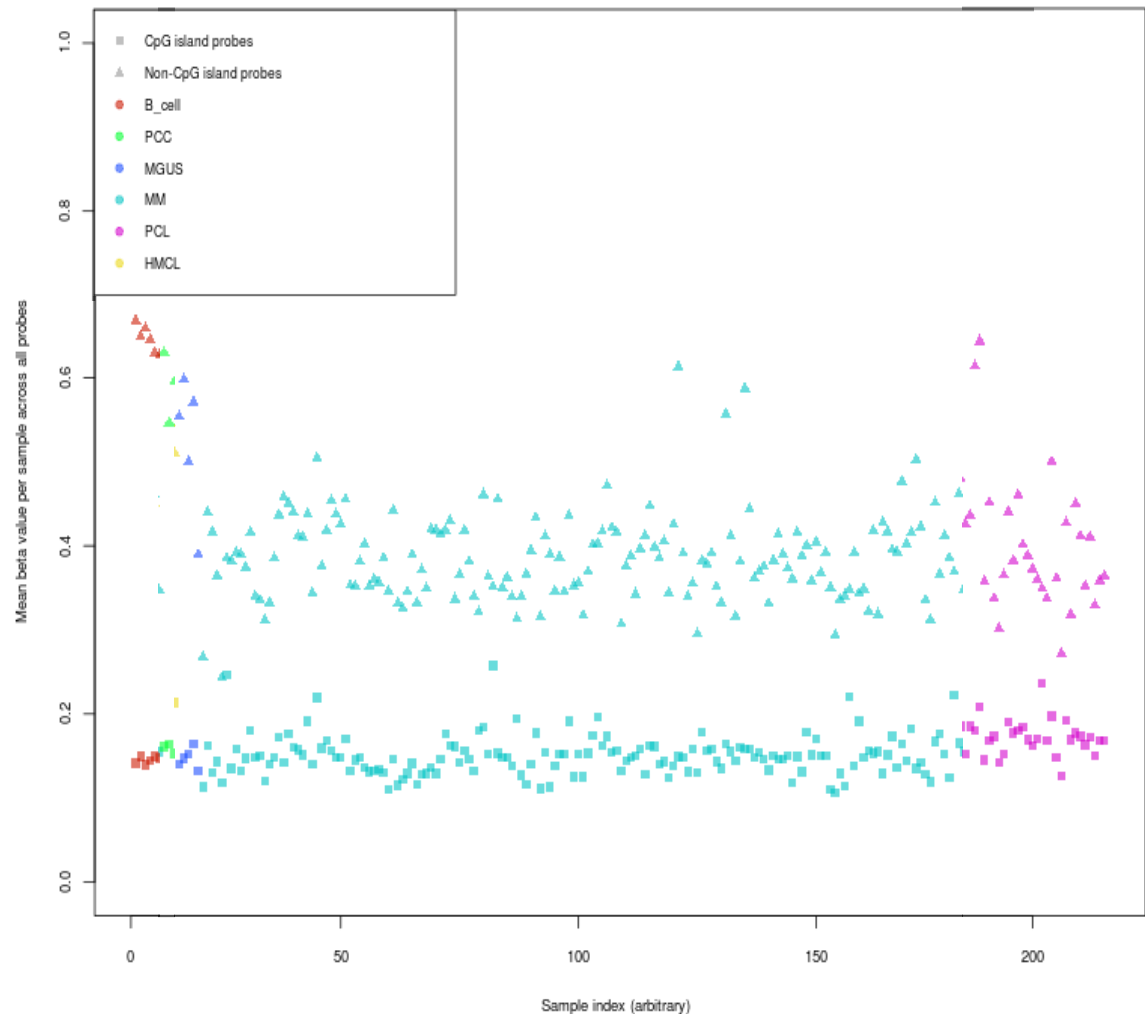
4620 probes (16.7%)

2876 unique genes (23.3%)

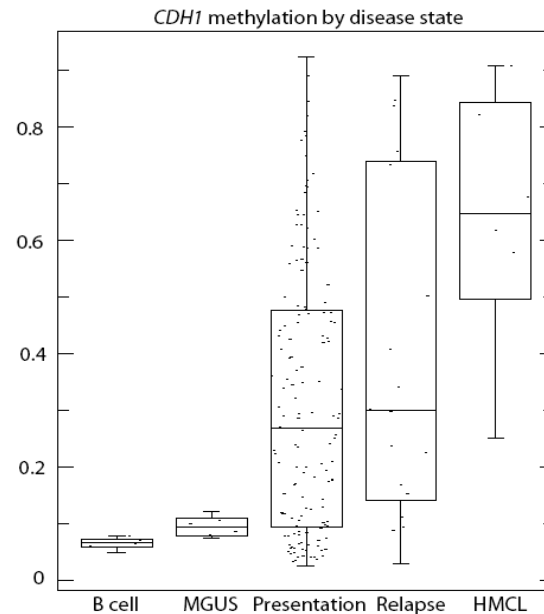
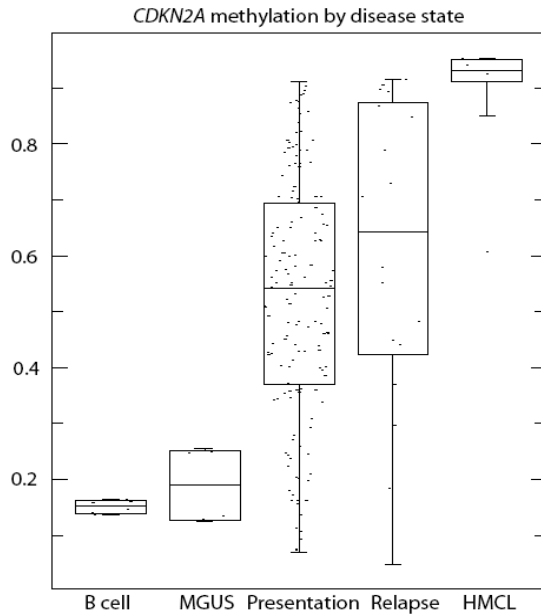
De-methylation of non-CpG island probes

- Genome Hypo-methylation is restricted to non-CpG island probes (▲) and not CpG island probes (■)

- Indicative of global hypomethylation and genome instability



MGUS → MM: Gene Specific hypermethylation



Hypermethylation of tumor suppressor genes

Negative regulation of cell cycle

(58 genes; *CASP2*, *CDH1* (*E-cadherin*), *CDKN2A* (*p16*), *CDKN2B* (*p15*), *CDKN1C* (*p57*), *DCC*)

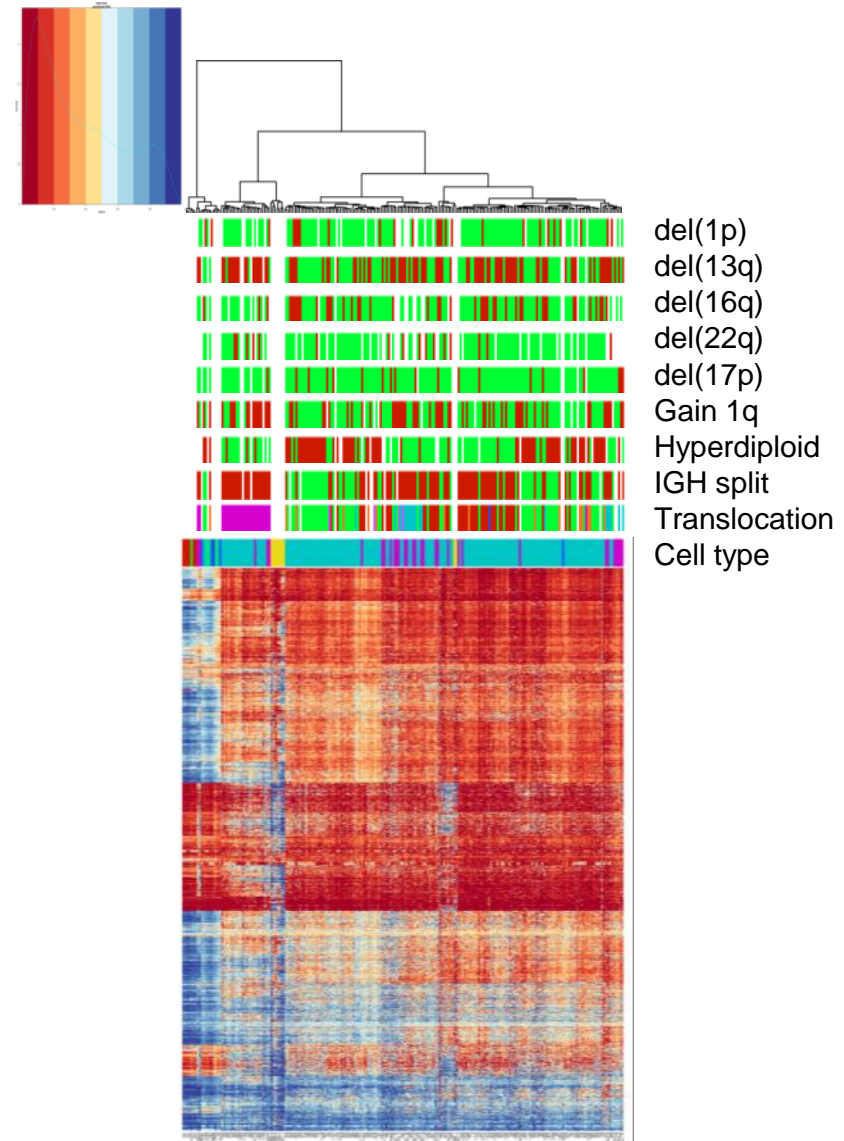
Cell adhesion molecules (152 genes, $P=1.3 \times 10^{-10}$)

Development (61 genes, $P=7.9 \times 10^{-18}$)

Are there cytogenetic specific methylation groups?

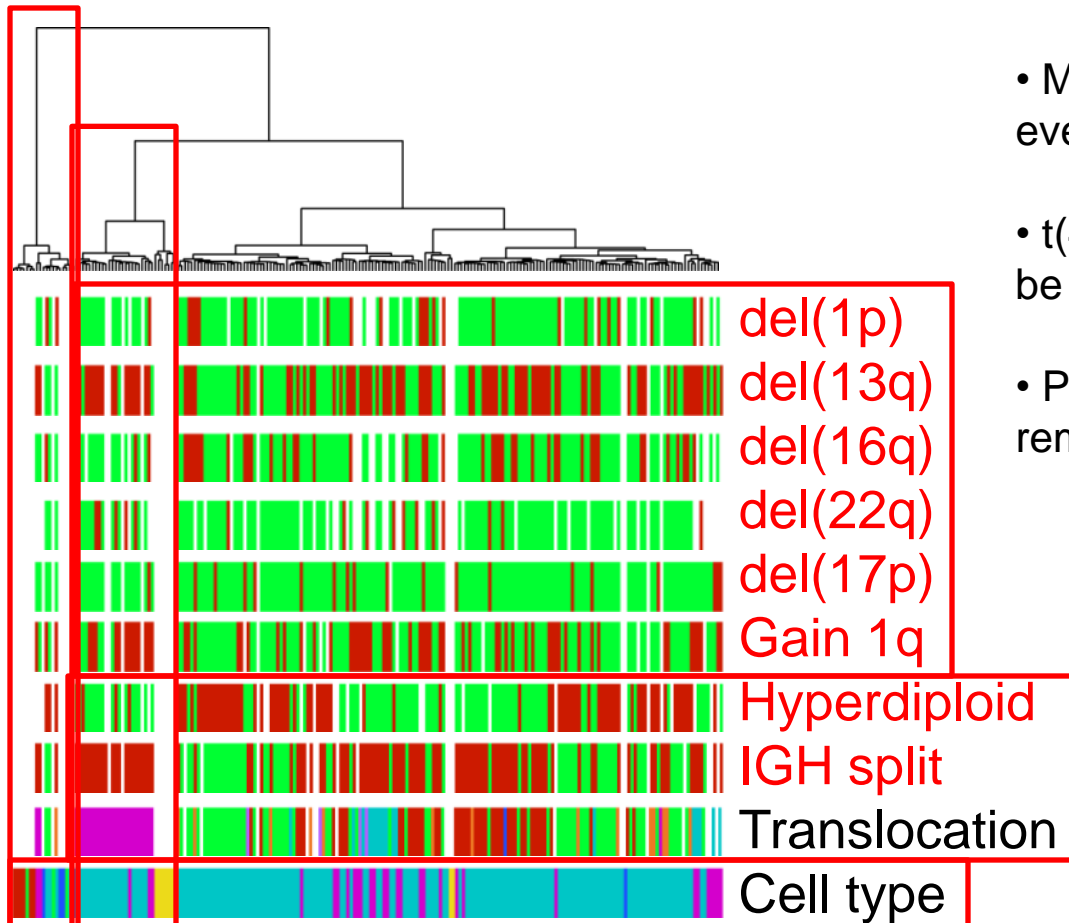
Unsupervised clustering of methylation data by individual samples

Annotated by copy number changes, hyperdiploidy, translocation and disease time point



Methylation status is defined by primary events

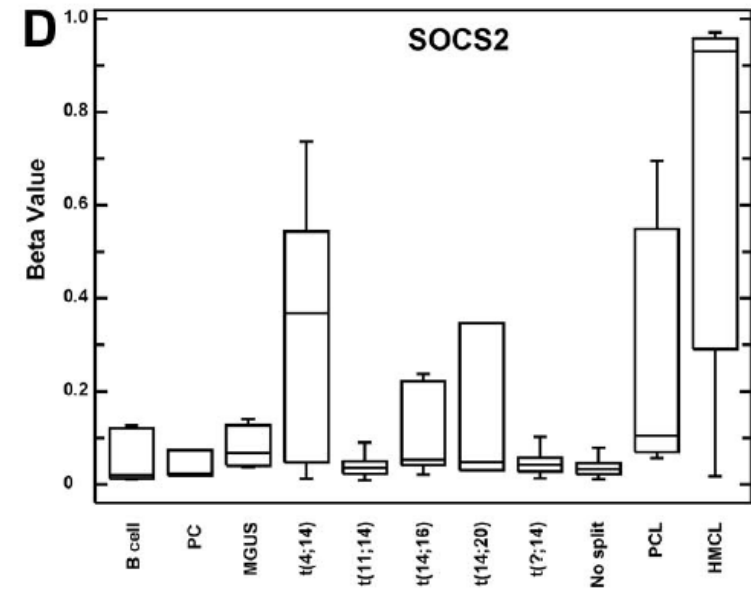
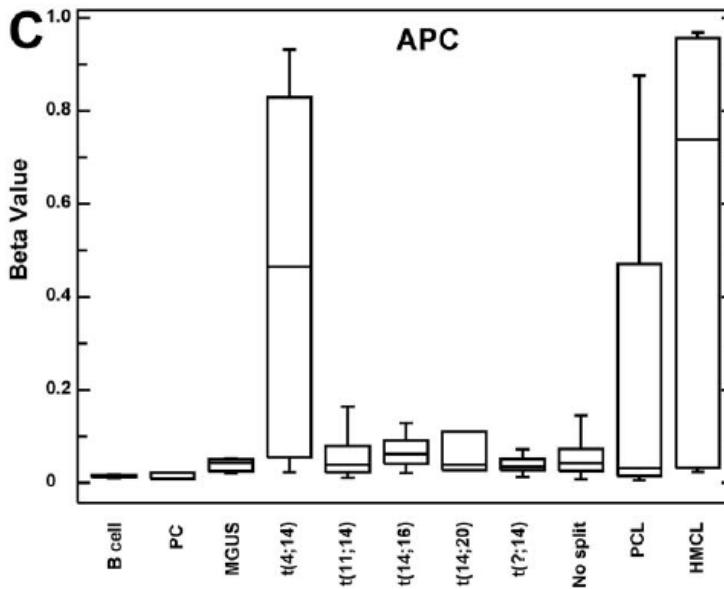
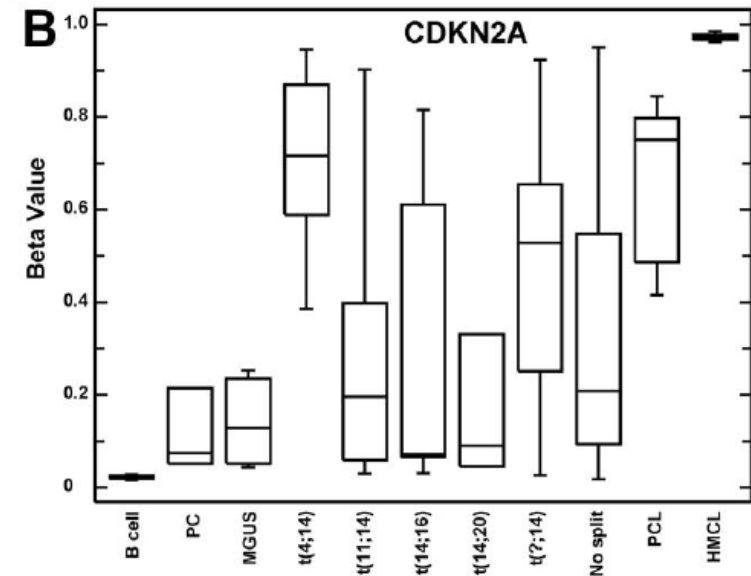
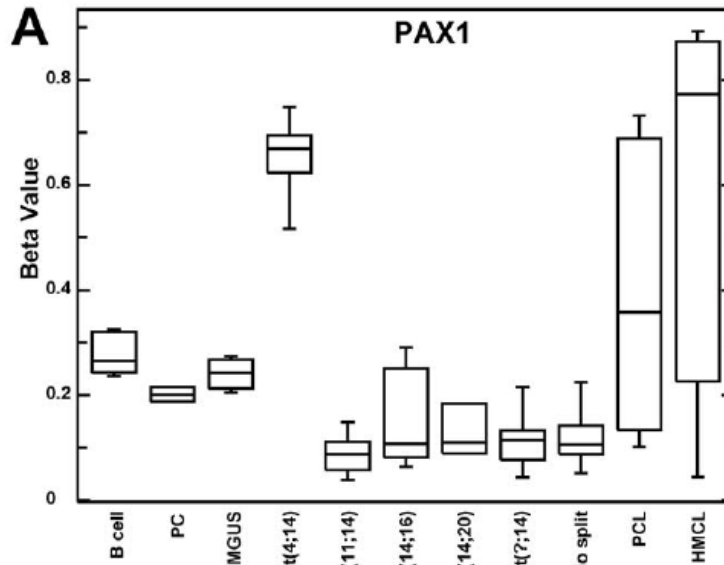
- Non-malignant cells cluster separately
- Copy number abnormalities do not cluster methylation patterns
- Methylation clustering is defined by primary events – translocations and hyperdiploidy
- t(4;14) samples are most different, and may be due to MMSET over-expression
- PCL samples do not cluster separately, but remain within their cytogenetic group



t(4;14), t(11;14), t(6;14), t(14;16), t(14;20)

B cell, NPC, MGUS, MM, PCL, HMCL

t(4;14)-specific CpG Hypermethylation

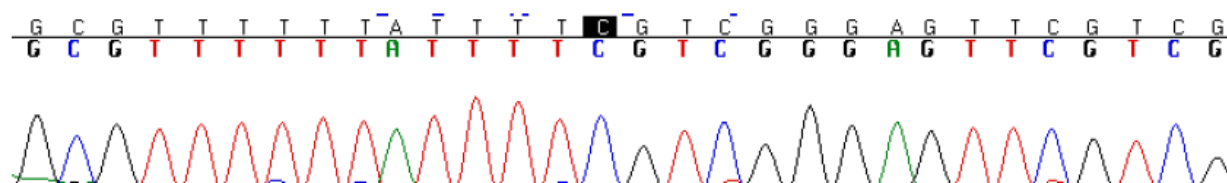


Validation of Array Results By BSP

APC

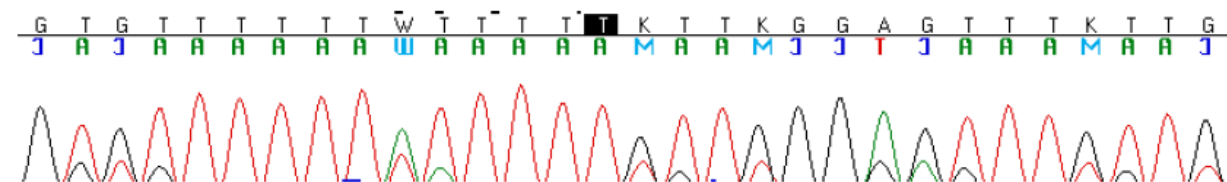
β -value

t(4;14)



0.57

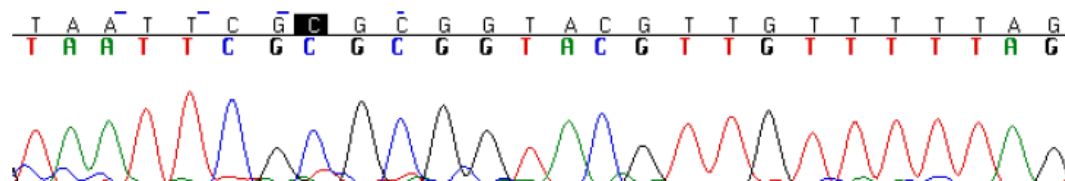
No split



0.07

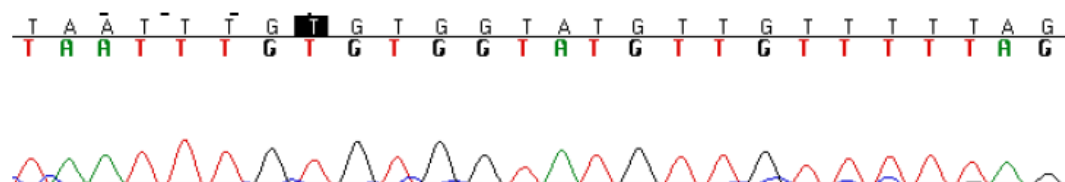
SOCS2

t(4;14)



0.54

No split



0.04

t(4;14) myeloma

Pathway analysis of hypermethylated genes in t(4;14) enrichment for:

- cellular development (P=2.6x10⁻²⁹)
- cell signalling (P=6.1x10⁻²⁷)
- cellular adhesion (P=7.3x10⁻²⁵)

GO#	GO term	Gene Count	%	P value
GO:0032501	~multicellular organismal process	503	33.18%	4.63x10 ⁻⁴⁴
GO:0048731	~system development	268	17.68%	2.57x10 ⁻²⁹
GO:0048856	~anatomical structure development	307	20.25%	8.28x10 ⁻²⁹
GO:0007275	~multicellular organismal development	325	21.44%	2.85x10 ⁻²⁸
GO:0007267	~cell-cell signaling	137	9.04%	6.13x10 ⁻²⁷
GO:0022610	~biological adhesion	147	9.70%	7.33x10 ⁻²⁵
GO:0007155	~cell adhesion	147	9.70%	7.33x10 ⁻²⁵
GO:0006952	~defense response	122	8.05%	8.87x10 ⁻²⁵
GO:0048513	~organ development	202	13.32%	1.63x10 ⁻²³
GO:0032502	~developmental process	388	25.59%	3.55x10 ⁻²¹
GO:0009653	~anatomical structure morphogenesis	169	11.15%	3.16x10 ⁻¹⁷

t(4;14) myeloma – correlation of methylation and expression

Gene	Chr.	Probe	Expression changes (U133 Plus 2.0)				Methylation changes (humanmethylation27)						Description
			t(4;14)	No split	Fold change	P	Probe	CpG Island	t(4;14)	No split	Difference	P	
<i>C20orf103</i>	20	219463_at	8.038	10.772	-6.656	.008991	cg09119967	False	0.472	0.231	0.241	.001135	Chromosome 20 open reading frame 103
<i>CD79A</i>	19	1555779_a_at	7.168	8.408	-2.362	.006872	cg04790874	False	0.484	0.222	0.262	4.11 × 10 ⁻⁵	CD79A
		205049_s_at	7.652	8.722	-2.100	.040305							
<i>FAM49A</i>	2	208092_s_at	6.461	7.775	-2.487	.009265	cg10106284	False	0.287	0.167	0.12	.033294	Family with sequence similarity 49, member A
		209683_at	5.631	6.891	-2.396	.019702							
		230276_at	7.031	7.898	-1.823	.023463							
<i>GLTSCR2</i>	19	234339_s_at	10.806	12.216	-2.658	1.05 × 10 ⁻⁷	cg16791686	True	0.307	0.159	0.148	3.36 × 10 ⁻⁵	Glioma tumor suppressor candidate region gene 2
		217807_s_at	13.126	14.083	-1.942	1.06 × 10 ⁻⁷							
<i>GPX1</i>	3	200736_s_at	9.846	11.311	-2.760	.000267	cg06613840	True	0.442	0.177	0.265	2.46 × 10 ⁻⁸	Glutathione peroxidase 1
							cg15900980	True	0.386	0.082	0.304	1.16 × 10 ⁻⁶	
<i>GRM8</i>	7	216992_s_at	4.346	4.896	-1.464	.002383	cg09868882	False	0.532	0.267	0.265	4.34 × 10 ⁻⁸	Glutamate receptor, metabotropic 8
<i>MAB21L1</i>	13	206163_at	3.443	4.138	-1.619	.037692	cg05093686	True	0.538	0.207	0.271	4.86 × 10 ⁻⁷	mab-21-like 1
							cg12029639	True	0.560	0.225	0.335	1.20 × 10 ⁻⁷	
<i>MBP</i>	18	209072_at	6.539	7.032	-1.407	.000475	cg12555907	True	0.841	0.411	0.43	2.61 × 10 ⁻⁸	Myelin basic protein
<i>NME4</i>	16	212739_s_at	8.007	8.658	-1.570	.012902	cg18676162	True	0.321	0.178	0.143	.027662	Nonmetastatic cells 4
<i>OSBPL10</i>	3	231656_x_at	5.979	6.995	-2.023	.002011	cg15840985	True	0.329	0.170	0.159	.000222	Oxysterol binding protein-like 10
<i>RPS2</i>	16	212433_x_at	13.275	14.107	-1.781	.000313	cg18279742	True	0.344	0.205	0.139	.041014	Ribosomal protein S2
		203107_x_at	13.825	14.543	-1.645	.000254							
		217466_x_at	10.499	11.145	-1.564	.001184							
<i>SOCS2</i>	12	203373_at	7.734	8.405	-1.593	.041561	cg04797323	True	0.450	0.140	0.31	.000257	Suppressor of cytokine signalling 2
							cg06630241	True	0.637	0.285	0.352	7.18 × 10 ⁻¹⁰	
							cg11738543	True	0.353	0.103	0.25	5.05 × 10 ⁻⁵	
							cg23412850	True	0.330	0.059	0.271	4.24 × 10 ⁻⁶	

Methylation and Expression Changes at the transition from Myeloma to Plasma Cell Leukaemia

Comparing methylation and expression changes

Methylation

161 MM samples vs 31 PCL samples



$P < 0.05$

1573 probes
(1394 genes)

Expression (U133 Plus 2.0)

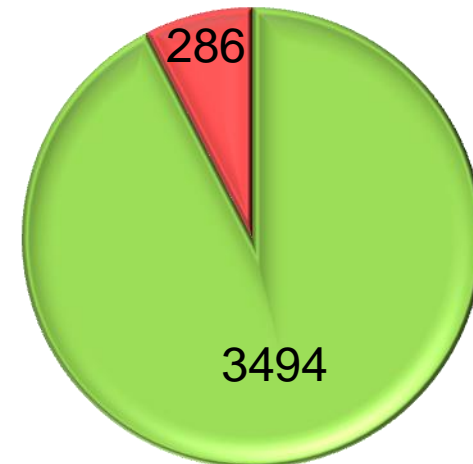
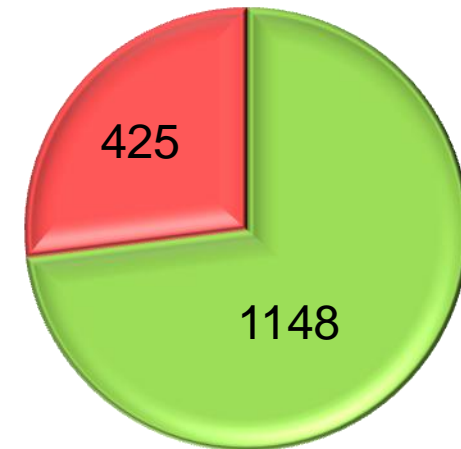
205 MM samples vs 10 PCL samples



$P < 0.05$, fold change > 2
Benjamini-Hochberg correction
3780 probes
(2971 genes)

In PCL samples

▲ increased
▲ decreased



Pathway analysis of changes in PCL (Ingenuity)

Methylation

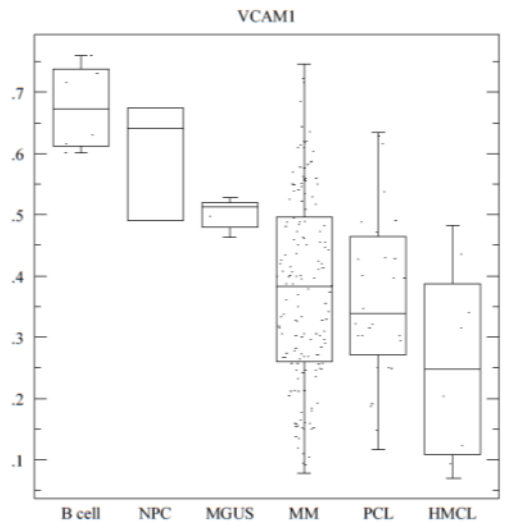
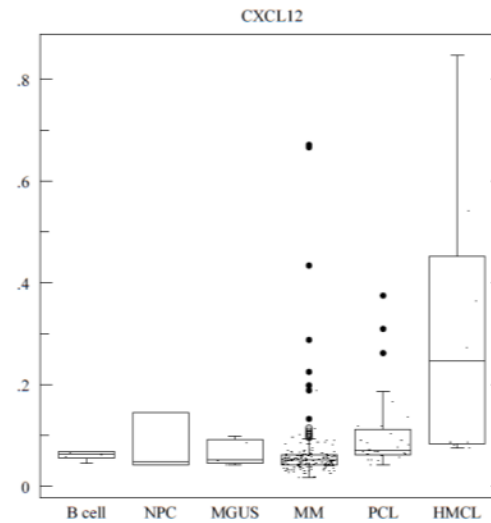
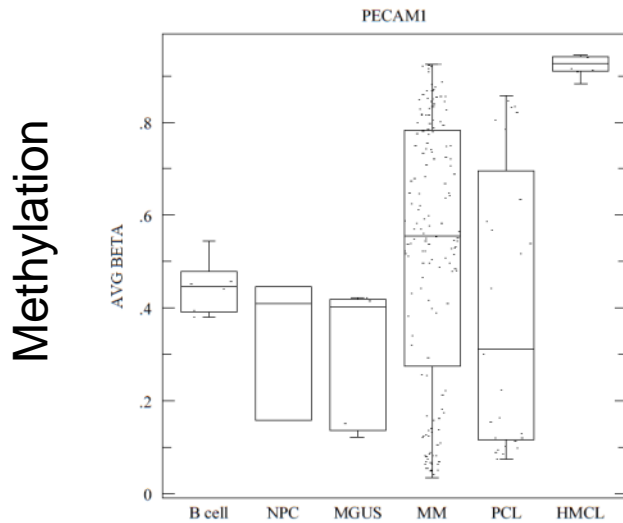
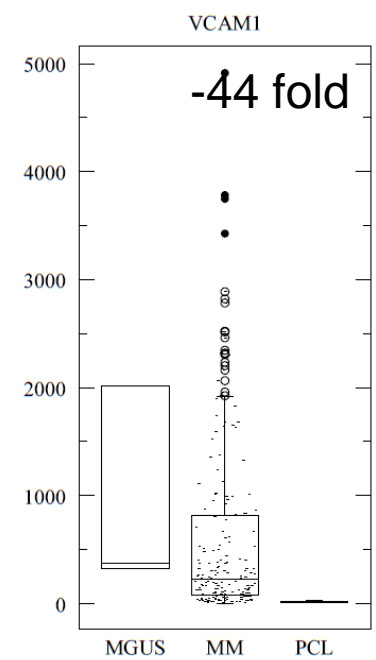
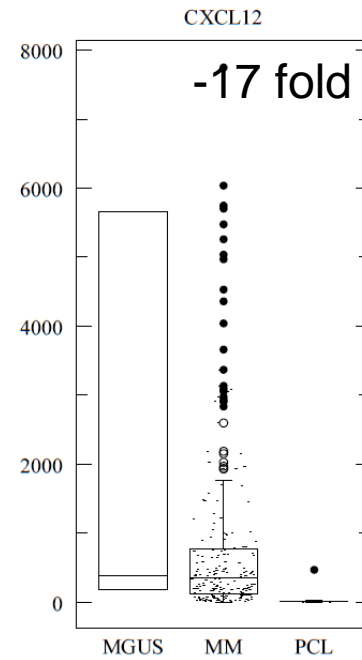
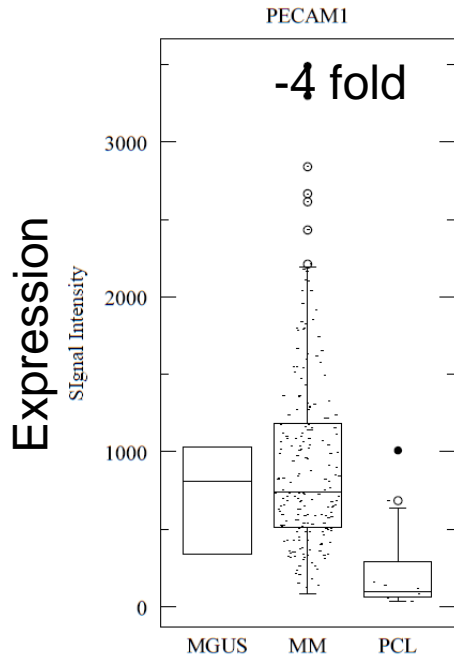
Top Networks		
ID	Associated Network Functions	Score
1 View	Cell Signaling, Nucleic Acid Metabolism, Small Molecule Biochemistry	47
2 View	Cancer, Cell Death, Skeletal and Muscular Disorders	34
3 View	Cell Morphology, Hematological System Development and Function, Hematopoiesis	32
4 View	RNA Post-Transcriptional Modification, Cancer, Cell Cycle	32
5 View	Cell-To-Cell Signaling and Interaction, Tissue Development, Cellular Movement	30

Expression

Top Networks		
ID	Associated Network Functions	Score
1 View	Cellular Movement, Amino Acid Metabolism, Post-Translational Modification	36
2 View	Cell Morphology, Cellular Function and Maintenance, Connective Tissue Development and Function	34
3 View	RNA Post-Transcriptional Modification, Molecular Transport, RNA Trafficking	34
4 View	Gene Expression, Post-Translational Modification, Genetic Disorder	34
5 View	Post-Translational Modification, Genetic Disorder, Metabolic Disease	32

Cellular movement is relevant to PC egress from the bone marrow into the peripheral circulation

Cell Adhesion Molecules are down-regulated in PCL samples



Integration of methylation and expression changes in MM and PCL



26 genes \uparrow expression and \downarrow methylation
9 genes \downarrow expression and \uparrow methylation

Methylation of genes affects expression

Expression fold change >1.5 , $P < 0.05$, difference >100

Methylation $P < 0.05$, difference >0.2

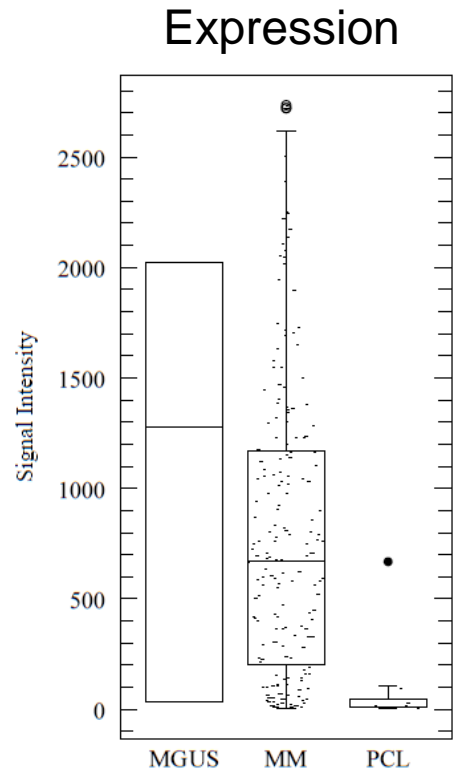
Decreased expression with increased methylation in PCLs

ACP1, ARIH2, CAV1, GLTRSC2, KIAA0652, QPRT, SLAMF1, SLC35B3, SPAG4, TPD52

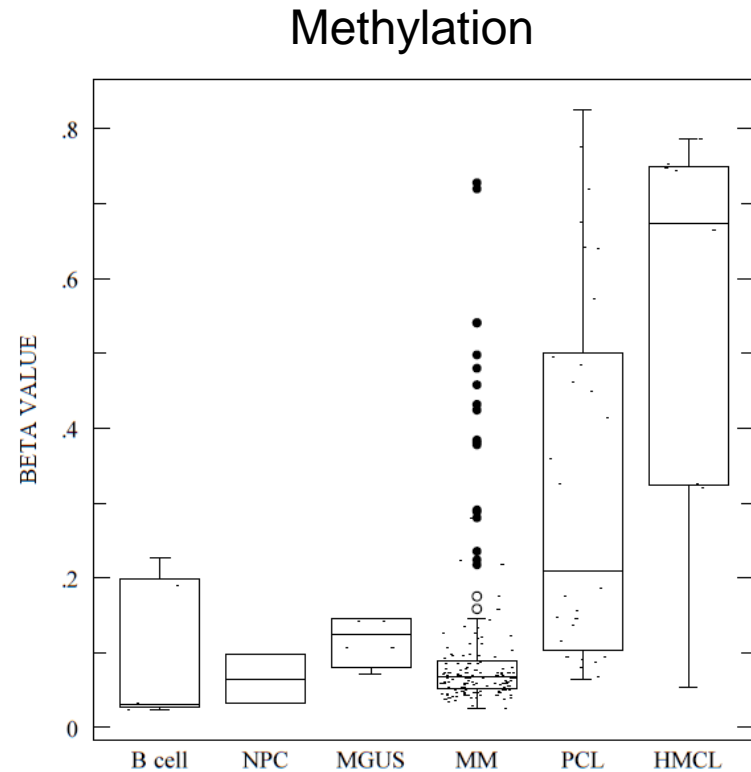
Increased expression with decreased methylation in PCLs

AP2A2, BANF1, BMP8B, C3orf63, C9orf82, CASP8, CCND1, CHAC1, CX3CR1, DTNBP1, GNPAT1, HUS1, IL10, LYAR, PSD4, RUNX2, SETD4, SLC7A11, SNRPB2, SNTB1, SPN, THUMPD1, TMEM41A

SLAMF1 (CD150) expression decreases as methylation increases



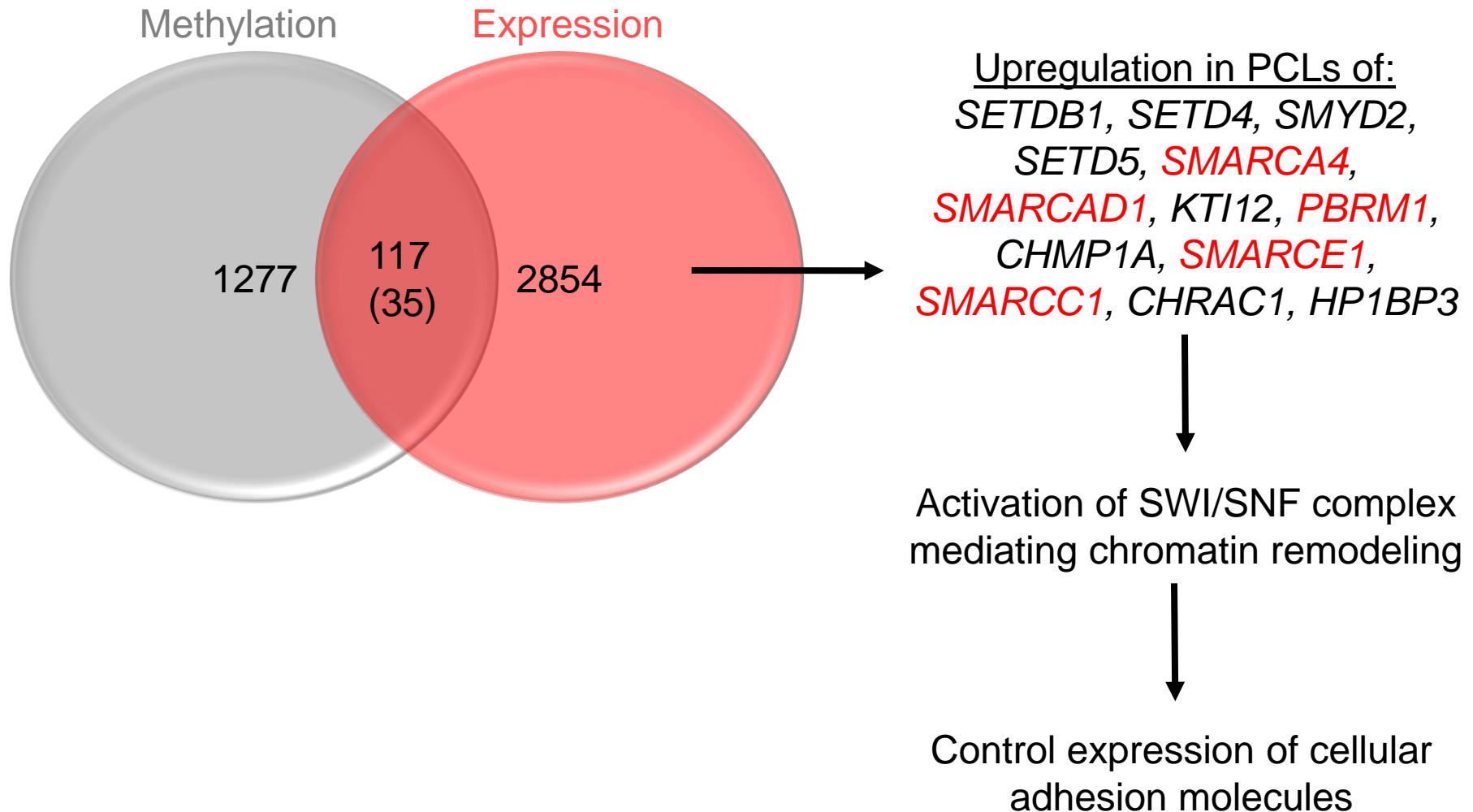
Fold change -7.9
P=0.03



Difference 0.25
P<0.001

Changes in expression of chromatin remodeling

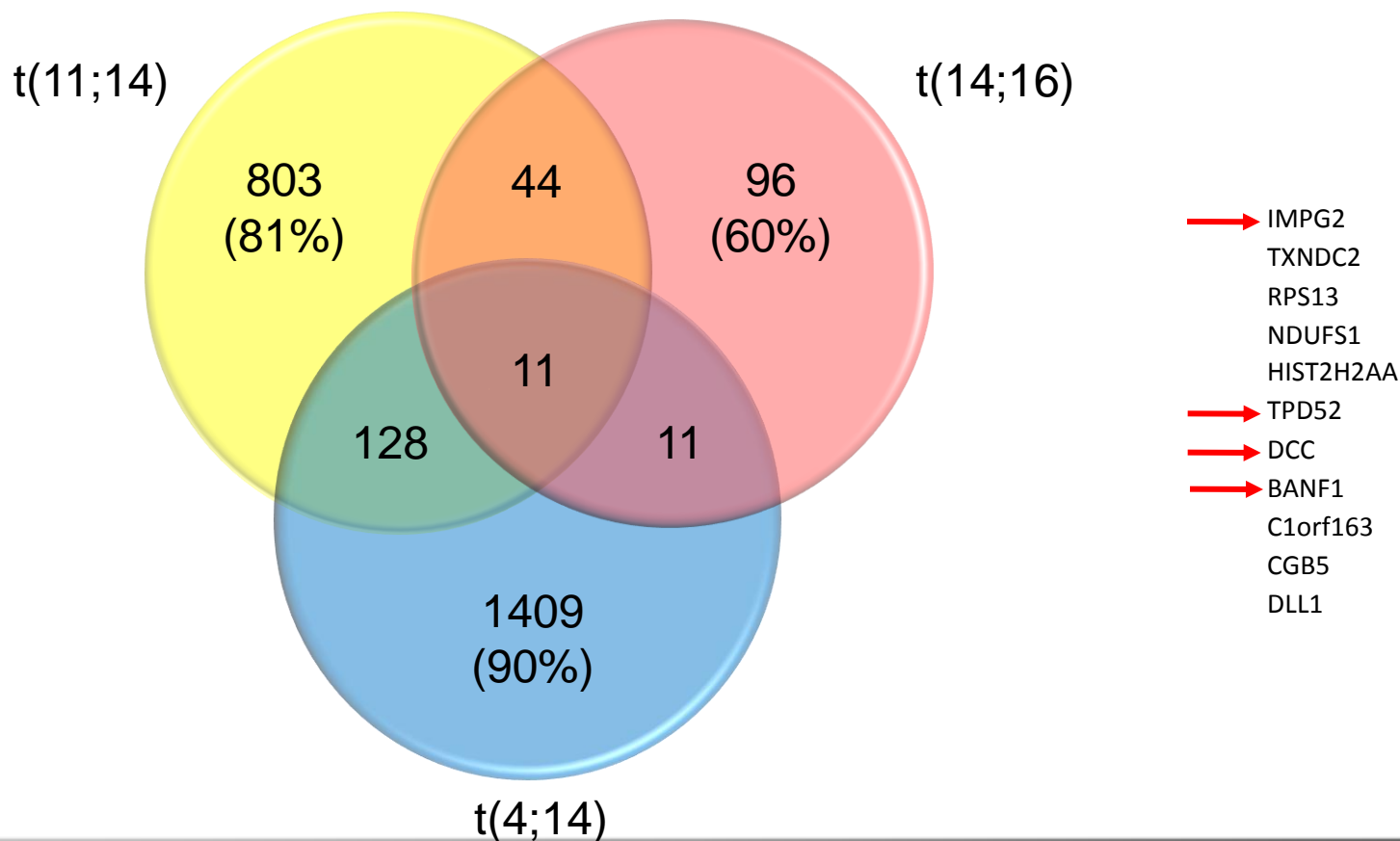
As DNA methylation does not directly control cell adhesion molecules, are there other mechanisms at work?



Methylation changes from MM to PCL

As PCL samples cluster within the cytogenetic group to which they belong, we performed translocation-specific methylation analysis between MM and PCL

t(11;14) 36 MM vs 9 PCL – 986 probes (882 increased methylation)
t(14;16) 7 MM vs 10 PCL – 162 probes (70)
t(4;14) 22 MM vs 5 PCL – 1559 probes (1553)



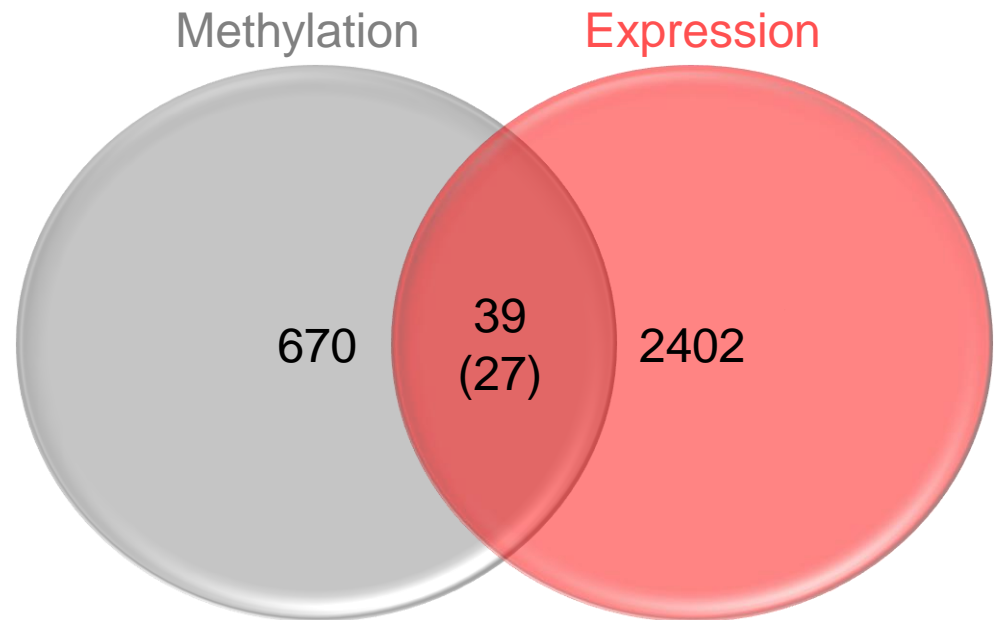
t(11;14)-specific methylation changes

Methylation

t(11;14) 36 MM vs 9 PCL

Expression

t(11;14) 34 MM vs 7 PCL



Decreased expression with increased methylation in PCLs

ARIH2, CAV1, CLC, CPEB4, CTGF, FSTL1, GNAS, GPR125, LPHN2, MBNL2, MMP8, MPO, P2RX1, PDE4B, PEBP1, PELI1, PLD4, SLAMF1, SNX9, TF, TPD52
Fold change -1.96 to -41.74

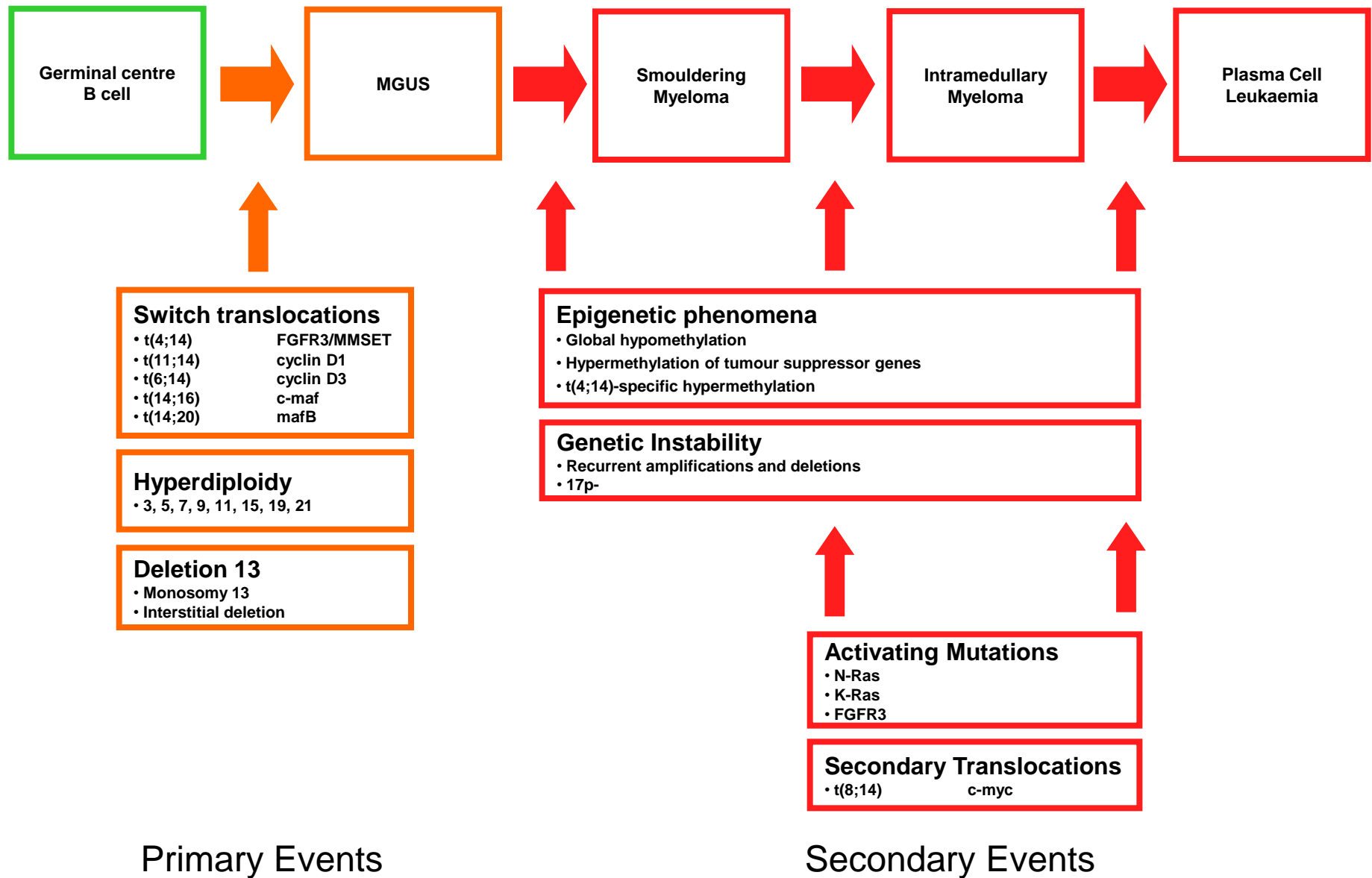
Increased expression with decreased methylation in PCLs

BLZF1, CASP8, MVP, PARP12, PDE4DIP, RUNX3
Fold change 1.75 to 4.34

Conclusions

- DNA methylation can be used to define myeloma cytogenetic subgroups and disease state
- MGUS>MM transition is characterised by genome-wide hypomethylation and gene-specific hyper-methylation
- t(4;14) samples have a more pronounced DNA hypermethylation signature which may be linked to MMSET over-expression
- copy number abnormalities do not drive clustering of methylation data, but primary events (translocations and hyperdiploidy) do
- PCL samples do not cluster separately, but instead remain within their original cytogenetic subgroups indicating fewer methylation changes at this transition
- Changes between MM and PCL are characterised by an increase in DNA methylation and a dramatic change in gene expression
- Decreased expression of CAMs in PCL samples is not associated with increased DNA methylation at their genomic location

Disease Stages and Genetic Events



Acknowledgements

ICR

Prof Gareth Morgan
Faith Davies
David Gonzalez
Christopher Wardell
Kevin Boyd
David Johnson
Paola Leone
Matthew Jenner
Emma Smith

Salisbury

Fiona Ross
Laura Chiecchio

Milan

Antonino Neri

Aalborg

Mette Nyegaard

