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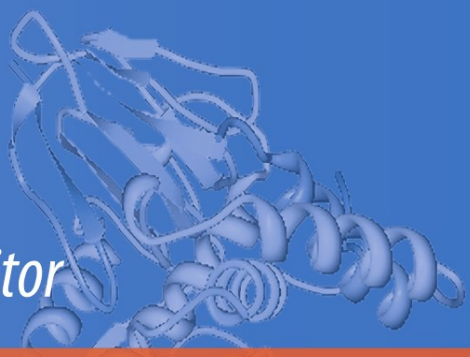

Tapas K. Kundu *Editor*

Epigenetics: Development and Disease

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Epigenetics: Development and Disease

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Tapas K. Kundu
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Epigenetics: Development and Disease

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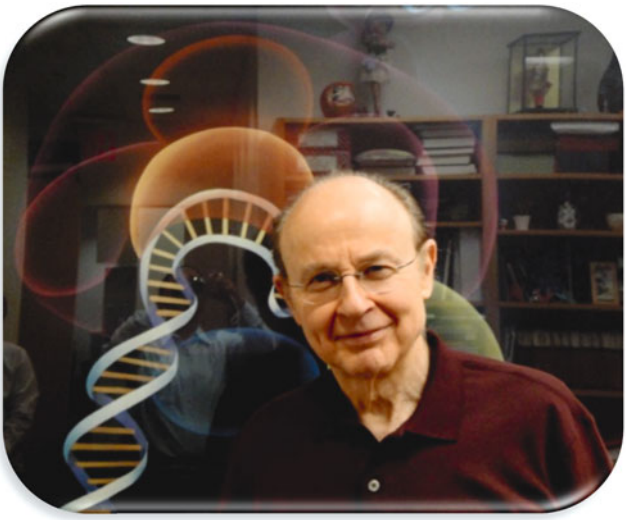
To
A teacher, a mentor and a source of constant inspiration, ***Prof. G. Padmanaban****, distinguished biotechnologist and former Director of Indian Institute of Science, Bangalore-12, India.*

Foreword



"Our interest in and understanding of the concept of epigenetics has increased dramatically in the past decade, with the general perception of epigenetics having evolved from one of a phenomenon considered to originate from anomalous and disparate patterns of inheritance to one that is linked to a variety of normal and disease-related physiological processes through specific molecular mechanisms. Notably in this regard, the field of epigenetics has come a long way from an early predominant emphasis on DNA methylation to the current inclusion of chromosomal histone modifications and, more recently, non-coding RNAs in epigenetic regulatory events.

Epigenetic phenomena are intimately related to chromatin structure and organization and thereby influence gene expression. The importance of epigenetics for development and cell differentiation is increasingly clear and underlying mechanisms

are being unraveled. Similarly, epigenetic changes are now being linked to early events in the pathogenesis of diseases such as cancer, diabetes, and many others. These revelations have sparked efforts to develop new generation therapeutics against components of the epigenetic machinery for the treatment of complex multifactorial diseases.

This collection entitled *Epigenetics: Development and Disease* very effectively covers the above-mentioned aspects of epigenetics research, along with considerations of the evolution of the epigenetic machinery and the role of epigenetics in transcriptional regulation, in five separate parts. The various chapters in these parts have been written by experts who themselves have contributed significantly to their respective fields. Although there are other books with similar titles, this book provides a comprehensive update on the role of epigenetics in development and disease, efforts to develop therapeutics for some of these diseases and the role of epigenetics in transcriptional regulation. Consideration of the latter topic is especially important in view of the probable key role of transcription factors in the initial induction or establishment of many epigenetic changes or states – as dramatically evidenced by the ability of small subsets of ectopic transcription factors to reprogram somatic cells to pluripotent states through epigenetic changes.

Last but not least, the editor, Tapas K. Kundu, himself is an active scientist in the field and deserves a great deal of appreciation for his excellent job in conceiving and bringing to fruition this book. Students, as well as established investigators, will find the book to be a stimulating overview of the field."

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Robert G. Roeder, Ph.D.
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Preface

The field of 'Epigenetics' has moved on from the Waddington concept proposed in the year 1942; the definition has undergone a constant expansion and the scope of this subject has broadened over the years. The actual resurrection of the field can be marked by the discovery of the first histone acetyltransferase, GCN5, in the year 1995 by David Allis' group. Although the activity of histone modifications (acetylation and methylation) and its role in the transcriptional activation was discovered by Vincent Allfrey in 1964 in a very elegant manner, its significance could only be appreciated by the scientific community after the identification of the GCN5 acetyltransferase and the subsequent expansion of the histone modifying enzymes family. Initially epigenetics and DNA methylation mediated gene regulation was thought to be synonymous. However, this concept has now been replaced with the understanding that these modifications along with DNA methylation form the basis of epigenetic phenomenon. However, all the histone modifications need not be involved at the same time in this event. Furthermore, it has also been realised that several non-histone proteins which can harbor the similar modifications such as acetylation and methylation also form an integral component of the epigenetic network.

I was fortunate to be associated with the growth of the field since 1996 during my days in the Roeder (Robert G Roeder) Laboratory in the Rockefeller University, where a majority of my work was towards understanding the mechanism of transcriptional regulation by histone acetyltransferase complexes and their recruitment in the activator dependent transcription from chromatin. Coincidentally, at the same time I was also a part of the discovery of the first p300 and PCAF acetyltransferase activity specific inhibitors (a collaboration with Philip Cole's group). It is during this time that I got the opportunity to interact with Vincent Allfrey, David Allis and Jerry Workman. Interaction with Vincent Allfrey was really memorable. Vincent's approach towards the discovery of histone modifications was really a bold step in the late 1960s when the use of radioactive material was difficult even at the Rockefeller University. However, Vincent was confident and optimistic about the histone modification field and its link to epigenetics. When I met him for the last time, it was coincident with Elizabeth Pennisi's article in *Science*, highlighting the discovery of acetylation; the last line of which read "Vincent Allfrey should be pleased".

I found that indeed Vincent was really happy. Vincent passed away soon in the year 2002.

Back home in India, I continued in the field, focussing on the regulation of chromatin dynamics by non-histone chromatin proteins, histone chaperones and also small molecule modulators of histone modifying enzymes. At this juncture, in 2007, I got an opportunity to edit a volume of Subcellular Biochemistry entitled 'Chromatin and Disease' (Vol. No. 41). While editing this volume I started realizing that chromatin function is tightly linked to epigenetic phenomenon and that the next volume must be on 'Epigenetics'. I thank the editorial board members, especially, Robin Harris and Dipak Dasgupta, who were so forthcoming and encouraging that I took the responsibility to edit the present volume entitled 'Epigenetics: Development and Disease'.

Epigenetics is not the monopoly of eukaryotes. During the course of evolution, as the genomic organization became more complex and evolved into systematically arranged chromatin structure, epigenetic machineries also started appearing as early as in Archaea. It is interesting to learn that soon after protozoans, all the four core histones along with different variants, ATP dependent remodeling systems and histone modifying enzymes are involved in genome function and thereby in the process of differentiation. In higher eukaryotes, epigenetically regulated dynamic chromatin function is the fundamental basis of differentiation and development. One of the basic cellular processes through which the epigenetic machineries operate is transcriptional regulation. Besides RNA Polymerase II driven transcription, RNA Polymerase I and RNA Polymerase III mediated transcription also requires histone modifications and promoter methylation. The non-coding RNA transcripts transcribed by RNA Polymerase III themselves function as one of the components of epigenetic machineries.

Cellular homeostasis is often disturbed in pathophysiological conditions. Thus in different diseases, inflammatory to infectious, epigenetic marks are altered during the disease progression. The consequent alteration of gene expression network is also remarkable. However, it is not yet established whether altered epigenetic marks are a cause or result of the diseased microenvironment. Nevertheless, the altered epigenetic marks are emerging as targets of new generation therapeutics, some of which are already in the advanced stages of drug development. Considering these facts, the present volume has been organized into five different parts: (i) Epigenetics and Evolution, (ii) Developmental Epigenetics, (iii) Epigenetics and transcription regulation, (iv) Epigenetics and Disease and (v) Understanding of Epigenetics: A Chemical Biology Approach and Epigenetic Therapy.

Experts from all over the globe (14 countries) have contributed excellent articles covering the thoughts expressed above. They have modified their article based on the reviewers' and my comments as and when they were requested, in spite of their heavily loaded schedule. I express my heartfelt thanks to all the contributors for their great effort. Several of my present laboratory colleagues and a few who have left the laboratory and gone abroad to pursue their further research career have contributed immensely to make this volume a reality, among whom I must acknowledge B Ruthrotha Selvi (presently at MRC HGU, Edinburgh, UK) and my present

lab colleagues Sujata Kumari and D. Karthigeyan. I also acknowledge Parijat Senapati and Snehajyoti Chatterjee without whose constant effort for more than a year, in the process of sending out invitation letters, time to time communications, organising the articles and giving several scientific inputs, publishing this volume would have been impossible. I, my research team as well as all the contributors greatly acknowledge all the reviewers who have worked so hard from behind the scenes for their valuable comments to improve each article.

This book is dedicated to Prof. G. Padmanaban, who is not only associated in all the scientific ventures I am involved in but has also played an active role in maturing the idea of the whole book through several discussions. I am at a loss of words to express my gratitude towards him. Last but not the least, I thank the past and present staff members of Springer, Max Haring, and Marlies Vlot, who worked hard with us to bring this volume for all of you. All the contributors and myself hope that this volume will be useful to students who are learning chromatin biology and epigenetics, the teachers and researchers of the field, and also scientists from pharmaceutical industries.

Bangalore-64 Tapas K. Kundu

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I thank the reviewers who spent their precious time and greatly contributed through their valuable comments on each chapter.

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Contents

Abbreviations

5-Aza-CdR	5-aza-2'-deoxycytidine
5hmc	5-hydroxymethylcytosine
5mC	5-methylcytosine
7SKsnRNP	7SK small nuclear ribonuclear protein
AAP	ambiant air pollution
ABC	ATP-binding cassette
AML	acute myeloid leukaemia
APC	antigen-presenting cell
APOBEC3G	Apolipoprotein B mRNA-editing enzyme-catalytic polypeptide-like protein 3G
APP	Amyloid beta (Aβ) precursor protein
AR	Androgen receptor
ARE	Antioxidant response element
ART	Anti-retroviral therapy
ASC	adult stem cells
ATF2	Activating transcription factor 2
ATM	Ataxia telangiectasia mutated
ATR	Ataxia telangiectasia related gene
ATRX	Alpha thalassemia/mental retardation syndrome X-linked
BBB-	Blood brain barrier
BRCA1	Breast cancer 1, early onset
BRG1	Brahma-related gene 1
CAF-1	Chromatin assembly factor-1
CAP	catabolite activator protein
CARM1	Coactivator-associated arginine methyltransferase 1
CAST	CD3 epsilon-associated signal transducer
CBF-1	C-promoter Binding Factor-1
CCL19	C-C motif chemokine 19
CCL21	C-C motif chemokine 21
CcrM	cell cycle regulated methyltransferase
CD	chron's disease

Cdk9	Cyclin-Dependent Kinase 9
CDKN2	Cyclin dependent kinase 2
CENP-A	Centromere protein A
CF	core factor
CHD7	Chromodomain helicase DNA-binding protein 7
ChIP	Chromatin immunoprecipitation
CNS	conserved non-coding sequence
CNS	central nervous system
CpG	cytosine-phosphate-guanine
CREB	c-AMP response element binding protein
CRFs	Chromatin reassembly factors
CSB	Cockayne syndrome B protein
CSC-	Cancer stem cell
CSP-	Carbon nanospheres
CTCF	CCCTC-binding factor
CTD	Carboxy-Terminal Domain
CTIP2	Chicken ovalbumin upstream promoter transcription factor interacting proteins 2
CTK7A-	Sodium 4-(3,5-bis(4-hydroxy-3-methoxystyryl)-1H-pyrazol-1-yl)benzoate
CTL	Cytotoxic T-Lymphocytes
CTPB-	N-(4-Chloro-3-trifluoromethyl-phenyl)-2-ethoxy-6-pentadecyl-benzamide
DAC	5-deoxy-azacytidine
Dam	DNA adenine methyltransferase
DAPK1	Death associated protein kinase 1
Dcm	DNA cytosine methyltransferase
DEP	diesel exhaust particles
DNMT	DNA methyltransferase
DNMT1/3A/3B/3L	DNA methyltransferase 1/3A/3B/3L
DR3	death receptor 3
DSB-	Double strand break
EGCG	(−)-Epigallocatechin gallate
EMT	epithelial-to-mesenchymal transitions
eNoSC	energy-dependent nucleolar silencing complex
EPC	epithelial progenitor cells
ER	Estrogen receptor
ESC	embryonic stem cells
ESCC-	Esophageal squamous cell carcinoma
ETS	external transcribed spacer
FACT	facilitates chromatin transcription
FGF	fibroblast growth factor
GADD45α	growth arrest and DNA damage inducible protein 45 alpha
GFP	Green Fluorescent Protein
GMCSF	Granulocyte macrophage colony stimulating factor

H2AK119u	ubiquitinated histone H2A at lysine 119
ubiquitinated	histone H2A at lysine 119
H3K27me3	trimethylated lysine 27 on histone H3
H3K4me1/2/3	mono-di-tri-methylated histone H3 at lysine 4
H3K64me3	trimethylated histone 3 at lysine 64
H3K9ac	acetylated histone H3 at lysine 9
H3K9me1/2/3	mono-di-tri-methylated histone H3 at lysine 9
H4K20me3	trimethylated histone 4 at lysine 20
HAT	histone acetyltransferases
HATi	histone acetyltransferases inhibitor
HCC-	Hepatocellular carcinoma
HDAC	Histone deacetylase
HDACi	histone deacetylase inhibitor
HDMs	histone demethylases
HEXIM1	HMBA Inducible protein 1
HEXIM2	HMBA Inducible protein 2
HIV-	Human immune deficiency virus
HMBA	Hexa Methylene Bis Acetamide
HMG	high mobility group
HMTs	histone methyltransferases
HP1α	Heterochromatin protein-1α
HPC	high CpG promoter
HS	hypersensitive sites
HSC	hematopoietic stem cells
IBD	inflammatory bowel disease
ICM	inner cell mass
IG	isogarcinol
IGS	intergeneic spacer
IKK	IκB kinase
IL	interleukin
INO80	Inositol-requiring protein 80
iPSC	induced pluripotent stem cells
ISWI	Imitation SWI
ITS	internal transcribed spacer
JMJD3	jumonji-domain-containing protein histone deacetylase 3
KAT-	Lysine (K) acetyltransferase
KDAC-	Lysine deacetylase
LARP7	La related protein
LAT	Latency associated transcripts
lncRNAs	long non coding RNAs
LPC	low CpG promoter
LSF	Late SV40 Factor
MBD	methyl-binding domain
MBD2	Methyl-CpG-binding domain protein 2
MBD3	methyl-CpG binding domain protein 3

MBP	myelin basic protein
MDMs	Monocytes derived macrophages
MEPCE	Methyl phosphate capping enzyme
MHC	major histocompatibility complex
miRNAs	microRNAs
MLL	Histone methyl transferase
MOZ-	Monocytic leukaemia zinc-finger protein
mRNAs	messenger RNAs
MS	multiple sclerosis
MZ	monozygotic
ncRNAs	non coding RNAs
NER	nucleotide-excision repair
NF-κB	Nuclear factor-κB
NFAT	nuclear factor of activated T cell
NK	natural killer
NOD	non obese diabetic
NoRC	nucleolar remodelling complex
NORs	nucleolar organiser regions
NPM-	Nucleophosmin
NSC	neural stem cells and progenitors
ORC-	Origin recognition complex
p300/CBP	E1A binding protein p300/CREB-binding protein
Paf1c	Polymerase-associated factor 1 complex
PAF53	RNA Polymerase I associated factor 53
PARP-1	poly-ADP-ribose-polymerase 1
PBMC	peripheral blood mononuclear cell
PCAF	p300/CBP-Associated Factor
PcG	polycomb group of proteins
PEPCK-	Phosphoenolpyruvate carboxykinase
PGC	primordial germ cells
PGC-1α	peroxisome proliferator activated receptor gamma coactivator 1
PIC	pre-initiation complex
PMA	Phorbol 12-Myristate 13-Acetate
Pol	RNA polymerase
PRC2	Polycomb repressive complex 2
PRMT	Protein arginine methyl transferases
PRMT6	Protein arginine methyl transferase 6
pRNA	promoter RNA
pTEFb	Positive Transcription Elongation Factor b
PTM-	Post translational modification
PTRF	Pol I and transcript release factor
r	ribosomal
RA	rheumatic arthritis
RbBp5	retinoblastoma-binding protein 5
RDR2	RNA-dependent RNA polymerase 2

RFX1	regulatory factor X 1
RISC	RNA induced silencing complex
R–M	restriction – modification
RNS	Reactive nitrogen species
RORγt	RAR-related orphan receptor γ
ROS	Reactive oxygen species
RSS	RNA silencing suppressor
SAHA-	Suberoylanilide hydroxamic acid
SAM	S-adenosyl methionine
SCF-β-TrCP	Skp1-Cul1-F- box ligase containing the F-box protein β-transducin repeat-containing protein (βTrCP)
SCID	Severe combined immunodeficiency
shRNA	Short-hairpin RNA
siRNA	Small interfering RNA
SIRT	sirtuin
SL1	selectivity factor-1
SLE	systemic lupus erythematosus
SNF2	sucrose non-fermentable 2 chromatin remodeller
SWI/SNF	SWItch/Sucrose nonfermentable
SWR	Swi2/Snf2-related ATPase
T1D	type 1 diabetes
TAF1A	TAF_I48
TAF1B	TAF_I63
TAF1C	TAF_I110
TAF1D	TAF_I41
TAFII250	TATA binding protein associated factor 250
TAFs	TBP-associated factors
TAP	Transporter associated with antigen presentation
TBP	TATA-box binding protein
TCR	T cell receptor
TET	ten-eleven-translocation
TGF-β	transforming growth factor beta
Th	T helper cell
TIF1A/B	transcription initiation factor 1A/B
TIMP-3	Tissue inhibitor of metalloproteinase 3
TNF-α	Tumor necrosis factor alpha
TRBP	TAR RNA binding protein
TRD	target recognition domain
Treg	regulatory T cell
TrxG	tritorax group of proteins
TSA	trichostatin A
TSS	Transcription start site
TTF-I	transcription termination factor
UBF	upstream binding factor
UC	ulcerative colitis

UCE	upstream control element
UPE	upstream promoter element
vmiRNA	Viral miRNA
VPA	Valproic acid
vsr	very short repair
WRN	Werner's syndrome helicase
WSTF	Williams syndrome transcription factor
WT	Wild-type
YY1	Ying Yang Protein 1
ZBG	zinc-binding group

Part I
Epigenetics and Evolution

Chapter 1
Chromatin Organization, Epigenetics and Differentiation: An Evolutionary Perspective

Sujata Kumari, Amrutha Swaminathan, Snehajyoti Chatterjee, Parijat Senapati, Ramachandran Boopathi, and Tapas K. Kundu

Abstract Genome packaging is a universal phenomenon from prokaryotes to higher mammals. Genomic constituents and forces have however, travelled a long evolutionary route. Both DNA and protein elements constitute the genome and also aid in its dynamicity. With the evolution of organisms, these have experienced several structural and functional changes. These evolutionary changes were made to meet the challenging scenario of evolving organisms. This review discusses in detail the evolutionary perspective and functionality gain in the phenomena of genome organization and epigenetics.

1.1 Introduction

Epigenetics is a phenomenon which operates beyond the information present in the DNA sequence. However, information underlying the DNA sequence also plays an important role in the organization of epigenetic elements. Therefore, DNA sequence elements such as repetitive elements especially CpG islands are the functional components of chromatin organization as well as epigenetic machinery. Different histones and nonhistone proteins are dynamic packaging components of the versatile genome. Covalent modifications of DNA and these proteins as well as ATP remodelling factors contribute to the storage, maintenance and propagation of epigenetic information. As a consequence of diverse evolutionary pathways, these DNA and

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protein elements have evolved gradually towards complexity to facilitate the highly specific signal response and genome function. To begin with, we shall discuss about DNA elements.

1.2 DNA Elements: The Thread of Life

The number of genes, amount of DNA and collinearity of evolutionary hierarchy remains an unsolved puzzle. In prokaryotes, DNA sequence information is maximally used, whereas in higher organisms there is a large amount of DNA which is apparently functionless. However, this concept is rapidly changing in light of recent discoveries of small nuclear RNAs. Although it is beyond the scope of this review to discuss evolution of DNA elements from prokaryotes to higher eukaryotes, however, to discuss epigenetic phenomena, it is important to understand evolution of CpG islands and methylation.

Prokaryotes are devoid of CpG islands. Even in lower eukaryotes there are no defined CpG islands. In cold blooded animals primitive type of CpG islands are observed. The higher vertebrates (warm blooded animals) have well defined CpG islands. CpG islands get methylated and demethylated. Methylation of CpG islands is the founding phenomenon of epigenetic operations. In the heavily methylated eukaryotic genome, CpG islands in promoter regions have been kept free of methylation which is an interesting mechanism of gene expression regulation.

CpG Islands (CGIs) are not just the presence of CpG dinucleotides. Characteristically, CGIs are around 1,000 bp stretches of DNA that possess more than 60% G+C base composition and are devoid of DNA methylation. In mammals 1% of the genome is CGIs. Interestingly, half of the mammalian CGIs are not associated with the annotated promoters and are thus termed "orphan" CGIs. Highly evolved CGIs are found in warm blooded vertebrates. The other eukaryotes, such as *Drosophila*, *C. elegans* and yeast donot have typical CpG Islands. However, since in these organisms, DNA methylation system is almost absent, CpG sequences are unmethylated throughout the genome. In cold blooded animals, 'CGI-like' sequences are observed. Although initial reports suggested that mouse genome has far fewer CGIs as compared to the human genome, recent data reveals that the number is almost similar (for human 25,495 and mice 23,201) (Deaton and Bird 2011).

CGIs play important roles in the genome organization. Several experimental data have shown that CGIs are relatively nucleosome deficient or possess a structure of transcriptionally active chromatin. For example, histones H3 and H4 are found to be hyperacetylated and histone H1 is almost depleted at CGIs. Furthermore, genome-wide studies have shown that H3K4me3 is a predominant mark of CGIs associated with promoter regions. This category of CGIs is generally unmethylated, but another type of CpG Island which are non-gene CGIs and contain AluI repeat elements, are generally methylated (Ohlsson and Kanduri 2002). These islands are transcriptionally silent and possess highly compact chromatin structure.

The existence of a new class of unique CGIs that are methylated on both alleles has also been reported. The unmethylated or differentially methylated CGIs are possibly not only important for the active transcription start site but may also be the nucleation site for other physiological phenomenon such as spermiogenesis (Kundu and Rao 1996). However, methylation of CGIs plays an important role in the compact chromatin organization and genomic instability.

CGIs are important elements for transcription regulation. Mammalian transcription factor binding sites are generally GC rich. Sixty percent human protein coding genes' transcription start sites are located in CGIs. All the mammalian housekeeping genes' promoters contain CpG islands at their 5′ end. Several tissue-specific genes also have CpG islands. CGIs at promoter sites are unmethylated. In a heavily methylated mammalian genome, how these CGIs are kept methylation free is not yet well understood. Interestingly, CGI containing promoters often lack TATA boxes. As in every case, there are exceptions to this generalization and the examples are α globin, MyoD1 and erythropoietin which are CGI containing promoters and possess TATA boxes (Juven-Gershon et al. 2008). CGI chromatin at the promoter sites is predominantly acetylated and H3K4me3 modified (Fig. 1.1a). The protein Cfp1, a component of Set1 complex, specifically binds to unmethylated CGIs, and thus trimethylates the lysine 4 of histone H3 (Voo et al. 2000; Lee and Skalnik 2005). Recent evidence suggest that the promoters of miRNAs also posses CGIs with similar epigenetic state based on the signals. Orphan CGIs, may also possess active chromatin characteristics during development, suggesting their yet undefined role in the development (Illingworth et al. 2010).

As CGIs are important for chromatin dynamics and transcription regulation, they have profound effect on the maintenance of pluripontency and stem cell differentiation. The divalent mark of H3K4me3 (activation) and H3K27me3 (repression) at CGIs is the hall mark of undifferentiated ES cells (Fig. 1.1c). During the process of differentiation, some genes become completely active and possess only active marks while some others are completely silenced acquiring H3K27me3 and DNA methylation at CGIs (Azuara et al. 2006). Mechanistically, polycomb group protein complex 2 (PRC2) mediates the H3K27me3 methylation (Fig. 1.1b). Hypothetically, the PcG protein Ezh2 (catalytic component of PRC2), recruits the DNA methyltransferase to silence the CGI area of the genome (Viré et al. 2006).

The aberrant methylation of CGIs is predominant in several cancers. Several tumor suppressor genes harbor cancer specific methylation at CGIs. However, cancer specific methylation is not the monopoly of tumor suppressor genes. It is yet to be established whether CGI methylation is the cause or result of malignancy. Along with CGI methylation, threefold over-representation of H3K27me3 at CGIs was observed in malignant colorectal cancers (Illingworth et al. 2010). Cancer specific methylation equally affect annotated promoters as well as orphan CGIs. Role of PRC2 complex is being implicated in cancer specific hypermethylation (Illingworth et al. 2010). An interesting question or possibility is emerging: Does the DNA methylation mediated "pseudo-pluripotent state" favour the indefinite proliferation ability of a cancer cell? The role of CGI methylation in cancer stem cells is yet to be elucidated.

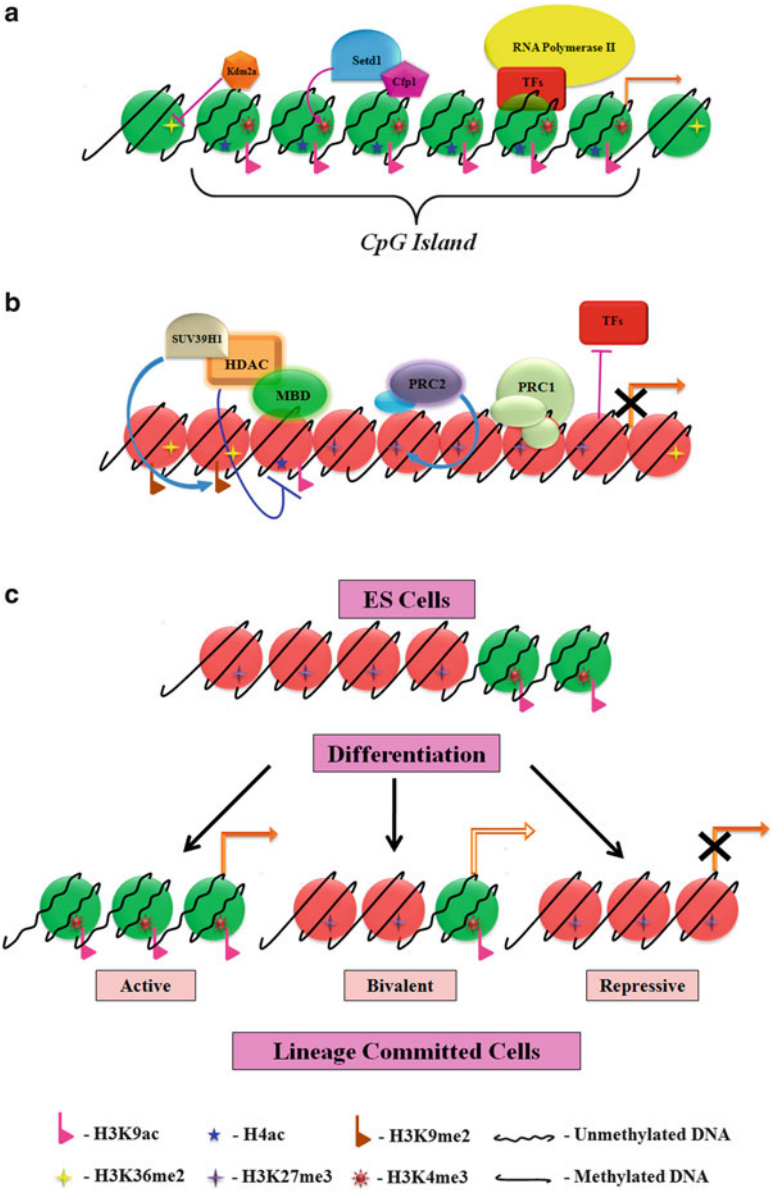

Fig. 1.1 CpG islands (CGIs) and transcription regulation. (**a**) Unmethylated CGIs in the promoter indicates transcriptionally active promoter. Cfp1 protein of Set complex binds to unmethylated DNA and trimethylated lysine4 of histone H3. At promoters bearing H3K4me3 and other active acetylation marks, RNA polymerase machinery is recruited which follows transcriptional activation. (**b**) Transcriptionally repressed promoter is characterized by methylated CGIs. Polycomb group protein complex 2 (PRC2) and other repressive complex containing histone deacetylases add repressive H3K27me3 marks and erase active acetylation marks respectively. (**c**) Undifferentiated ES cells possess divalent marks of H3K4me3 (*active*) and H3K27me3 (*repressive*) at CGIs. During lineage commitment, these marks are redistributed in such a way that required genes acquire active marks while others are silenced

1.3 Protein Elements (Histones and Histone Like Proteins)

There are different DNA binding proteins (predominantly sequence non specific binders) which compact the genomic DNA, not only to accommodate a huge amount of DNA in a tiny space but also to regulate gene expression.

1.3.1 Bacterial/Archeal Chromatin and Architectural Proteins

These proteins are basic in nature and belong to diverse classes. Histones are such proteins which form the basis of genome organization in eukaryotes. Histones have undergone evolution as histone fold motifs are also found in prokaryotic architectural proteins. It is now apparent that the relative amount of DNA bound proteins, presence/absence of histones and/or histone folds thereby affecting nucleosome stability have become principle criteria to resolve dichotomy between bacteria and higher life forms rather than counting for cellular architecture differences among them. Low protein:DNA ratio (<0.5) containing chromatin like structure is found in eubacteria, dinoflagellates, bacteriophage heads and mitochondria. The eubacterial chromatin is prone to aggregation and tends to form 'compactosome' (labile nucleosomes) (Kellenberger and Arnold-Schulz-Gahrnen 1992). This chromatin is very fragile and thus difficult to subject it to biochemical analysis. These DNA binding proteins lack histone folds. On the contrary, eukaryotic and archeal chromatin have higher protein:DNA ratio (>0.5) and the DNA is bound with regularly distributed protein partners and thus possesses more regular structure. Chromatin of *Halobacterium salinarium*, a halophilic archaebacterium was found to be composed of two types of DNA: protein free DNA and DNA assembled as nucleosomes forming fibers of 17–20 nm depicting the classical 'beads on a string' form (Shioda et al. 1989; Takayanagi et al. 1992). Chromatin compaction in non-nucleosome containing organisms like bacteria occurs via macromolecular crowding due to their higher concentrations in the cytoplasm (Zimmerman and Murphy 1996 and references therein; Bloomfield 1996). On the other hand, some organisms presumably utilize both nucleosomal packaging and crowding mechanisms at different time points in their life cycle to organize the genome (Drlica and Bendich 2000; Hildebrandt and Cozzarelli 1995).

1.3.1.1 Nucleoid Associated Proteins (NAPs)

E. coli nucleoid is irregularly shaped and poorly resembles the nucleus of higher eukaryotes. The nucleoid harbours genetic material organised as condensed circular chromosome having supercoiled domains. Nucleoid isolated from exponentially growing *E. coli* cells using a modified protocol showed fivefold more protein weight relative to DNA (Murphy and Zimmerman 1997). Apart from their well known DNA architectural roles, NAPs are also believed to act as buffers of DNA superhelical

structures (reviewed in Rimsky and Travers 2011). Different NAPs use different means to apply structural constraints on the DNA. Accordingly, these can be classified as DNA bridging and DNA bending proteins. In the following sections we shall discuss a few of these proteins in brief.

(a) **H-NS (Histone Like Nucleoid Structuring Protein)** is a non-specific DNA binding protein but shows preference to curved or bent DNA (Dame et al. 2001; Jordi et al. 1997). H-NS is a DNA bridging protein composed of two domains separated by a flexible linker. The C- terminal domain binds to DNA whereas the N-terminal domain is involved in dimerization. Dimeric H-NS bridges adjacent DNA duplexes (Dame et al. 2000, 2005a). The binding between DNA duplexes could be by the DNA binding domain of each monomer partner extending in opposite directions or the dimers can bind to the same strand of DNA (Badaut et al. 2002; Dorman 2004). Studies show that H-NS has approximately 1% genome coverage during exponential growth phase. *In vivo* evidence for architectural role of H-NS comes from over expression studies of H-NS which results in a compacted genome (Spurio et al. 1992).

(b) **SMC (Structural Maintenance of Chromosomes) complexes** are V shaped, and have large molecular weight (150–200 kDa) homodimers (Losada and Hirano 2005; Nasmyth and Haering 2005; Melby et al. 1998). The monomeric structure consists of two anti parallel coiled coils which join the extreme terminals of the protein. Two dimers can join through the apex region of the coils and form a V shaped dimer. This association is through a flexible hinge that provides the dimer an angular orientation. The globular region formed by association of the N and C terminus is called as the 'head' and contains the ATP binding domain. Dimers associate to form multimers usually yielding ring like structures or rossette structures. Stability of these structures depends on ATP binding and/or presence of protein co-factors (Hirano and Hirano 2004; Graumann 2001; Hopfner et al. 2000). The hinge region of SMC contains three consecutive lysine residues which probably help in DNA binding (Strunnikov 2006; Hirano and Hirano 2006). They associate with multiple DNA duplexes to yield these structures in the presence of different cofactors like ScpA, ScpB, MukE, MukF etc. These proteins indirectly contribute to chromatin organisation by affecting DNA topology (Lindow et al. 2002).

(c) **Lrp (Leucine-responsive regulatory protein)** is a small Leucine responsive protein with an N-terminal DNA binding domain (by virtue of three alpha helices) (Leonard et al. 2001) and leucine responsive C-terminus which are connected by a hinge region. Functional unit of Lrp is an octamer formed by four dimers resulting in a disc-like structure (Calvo and Matthews 1994; Brinkman et al. 2003). Lrp recognises consensus DNA binding sequence but can also bind with high affinity to sub-optimal binding sites (Cui et al. 1995). Modes of interaction of Lrps with DNA involves bridging mechanisms and wrapping of DNA around Lrp disc like structure remotely resembling the DNA-histone octamer interaction in the nucleosomes of higher eukaryotes (Beloin et al. 2003; Jafri et al. 1999; Thaw et al. 2006).

(d) **IHF (Integration host factor)** is an abundant non specific DNA bending protein. Its association with DNA can lead reduction in the length of DNA by 30% (Ali et al. 2001). It is composed of two subunits, a and b, which share a similarity

of upto 25% in their secondary structure. IHF is a heterodimer and its crystal structure reveals two flexible arms protruding out of the main body (Rice et al. 1996). A beta ribbon extends as an arm from the main body on both sides and inserts into the minor groove of DNA (Rice et al. 1996; Swinger and Rice 2004). The beta ribbon has a conserved proline residue which intercalates and causes hydrophobic interactions between DNA bases resulting in narrowing of the minor groove. IHF can cause sharp bends of upto 180° in DNA (Dame et al. 2005b; Dhavan et al. 2002; Rice et al. 1996; Lorenz et al. 1999). The negatively charged bent DNA is stabilised by the positively charged amino acid residues in the IHF main body (Swinger and Rice 2004).

(e) **HU (Histone-like protein from *Eschrichia coli* strain U93)** is a non specific DNA bending protein related to IHF. It is a dimeric protein and exists as two isoforms HUα and HUβ encoded by two homologous genes hupA and hupB in most bacteria. However, in enteric bacteria heterodimeric HUαβ is formed. Structurally, HU is similar to IHF in having a globular domain with two protruding beta ribbon arms (Swinger et al. 2003). The mode of interaction of HU with DNA is exactly the same except for the fact that they induce two kinks in different planes resulting in underwinding of the DNA double helices (Swinger et al. 2003). HU induced bends are flexible unlike IHF and can stabilize a range of different bend angles (Paull et al. 1993; Swinger and Rice 2004; Swinger et al. 2003). Owing to its random non specific DNA bending, overall DNA length reduction has been estimated upto 50% (Van Norrt et al. 2004). Another mode of HU- DNA association has been exemplified by *in vitro* studies where higher concentration of HU leads to its binding to DNA in a spiral manner forming stiff superhelical filaments (Van Noort et al. 2004). *In vivo* authenticity of this model is being addressed (Dame and Goosen 2002; Van Noort et al. 2004).

(f) **Fis (Factor for inversion stimulation)** is yet another DNA binding and DNA bending protein which is abundant in the early exponential phase. It functions as a dimer. Each monomer consists of four helices, two of which form the hydrophobic core body and the other two are involved in DNA bending (Johnson et al. 2005; Pan et al. 1996). It recognises a 15 bp consensus sequence but can also bind nonspecifically. Two arms of the Fis dimer bends DNA and fit themselves into the major groove. Fis binding sites are present at the upstream of many RNA operons where it acts as a transcriptional activator. Another mode of Fis DNA interaction is described as its binding to the DNA crossing point. At higher concentration it coats the DNA and results in DNA compaction (Schneider et al. 2001). Interaction between DNA bound to Fis dimers results in formation of DNA loops (Skoko et al. 2005). Thus Fis, via its DNA bending (non specific binding) and DNA looping properties contributes to nucleoid compaction.

(g) **DPS (DNA-binding proteins from starved cells)** As the name suggests, its role matches with its requirement during starvation. Its association with DNA protects it from UV light, redox insult and thermal shock (Minsky et al. 2002). DPS belongs to ferritin superfamily. It forms large oligomers like dodecamers with negatively charged surfaces. Interestingly, there are no DNA binding domains in the protein and due to negative charge of its oligomeric state is very unlikely to interact with DNA. Nevertheless, DPS binds to DNA non specifically.

A unique mode of DPS-DNA complex has been proposed. DPS-DNA complexes have been shown to form hexagonally packed 2-D arrays (Frenkiel-Krispin et al. 2004) with pores. These pores form a small positively charged patch by virtue of three lysine residues from three neighbouring N-termini of different dodecamers. This positive patch allows DNA interaction (Grant et al. 1998). 3-D arrays showing significant compaction have also been proposed via dodecamer-dodecamer interaction. Unlike eukaryotic histones which are exclusively involved in chromatin organization, none of the NAPs seem to be solely responsible for Nucleoid organization. Mutant studies show that these proteins can compensate for each other (Kano and Imamoto 1990; Paull and Johnson 1995) while some antagonize the other (Dame and Goosen 2002); For example DNA bending proteins (IHF, HU and Fis) locally antagonize effects of H-NS or Lrp.

Interestingly, NAPs dynamically appear and disappear based on the state of cellular life. This phenomena is also found in protozoans where highly differentiated cannonical histones and variants are involved. In each growth phase like early exponential, exponential and stationary phase, at least one of the DNA bending proteins predominates probably to antagonize the activity of other DNA binding proteins.

1.3.1.2 Archeal Histones

Most of the archeal chromosomal proteins are unique and do not share homology with bacterial proteins. Nevertheless, there are few exceptions. HTa protein isolated from *Thermoplasma acidophilum* is similar to HU family proteins (Oberto and Rouvie`re-Yaniv 1996; DeLange et al. 1981). HTa is a DNA binding protein that stabilizes DNA under thermal stress. *Sulfolobus* species possess different small, acid soluble, DNA binding polypeptides. These bind to minor groove of the DNA causing a bend and gets coated around the bent DNA (Robinson et al. 1998; Agback et al. 1998). DNA bending protein MC1 was identified from *Methanosarcina barkeri* which introduces kinks in the DNA without altering its contour length (Toulmé et al. 1995).

Archeal histones were first discovered in *Methanothermus fervidus* belonging to *Methanobacterium* clade (Sandman et al. 1990). Now it is known that almost all lineages of euryarchaeota possess histones (Sandman et al. 1997). Archeal histone-DNA assembly showed almost classical 'beads on a string' structure (Pereira et al. 1997; Sandman et al. 1990). Most of the archeal histones contain a single histone fold (represented by three alpha helices, α1-α3 and two intermittent loops, L1 and L2) (Fig. 1.2a). Archeal histones lack the N-terminal tail and C-terminal extensions or tails. There are two distinct histone like proteins in *M. fervidus* named as HmfA and HmfB. Archeal histones are grouped in two groups A and B based on their N-terminal residue. In archeal histones A, N-formyl methionine is replaced by alanine or glycine at position 1 whereas archeal histones B retain formyl methionine at position 1. Amino acid composition of HmfA and B differs which results in different tetramers

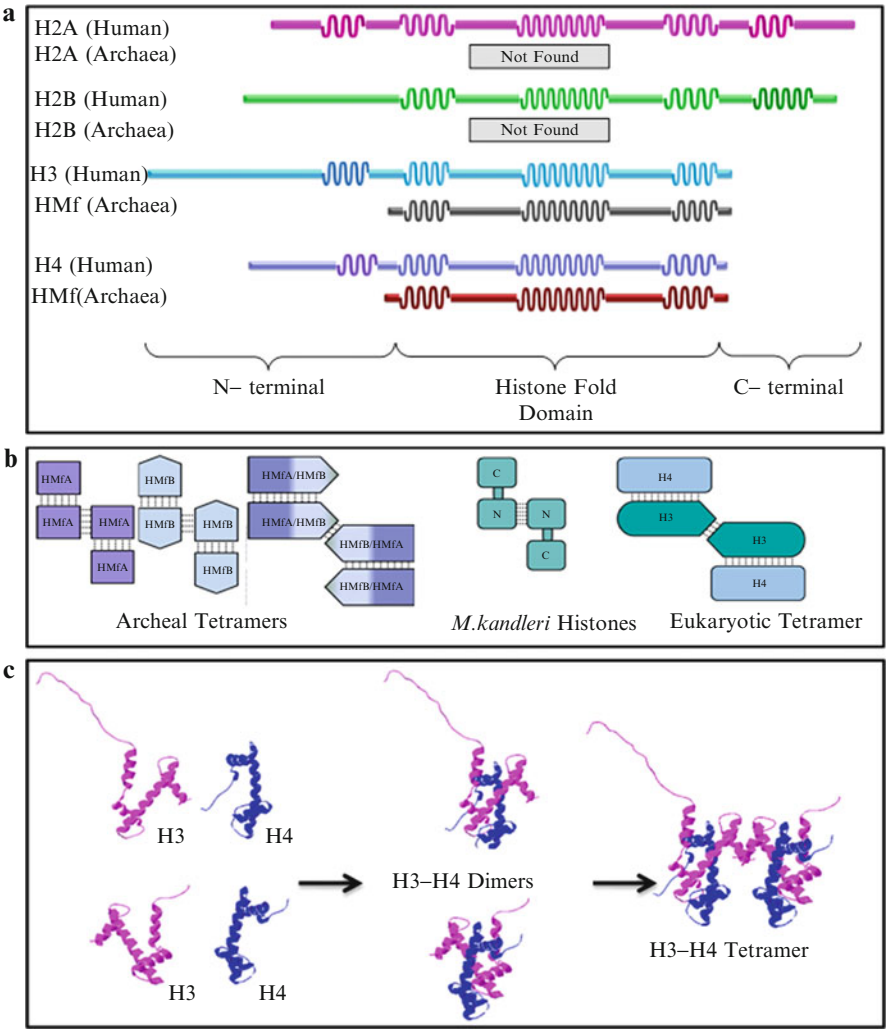

Fig. 1.2 Archeal and eukaryotic histones. (**a**) Line diagram of eukaryotic core histones depicting their histone fold domains and N terminal and C terminal extensions. (**b**) A proposed evolutionary route for transition from archeal to eukaryotic tetramers. *M. kandleri* (an archaebacterium) histones (doublet) are proposed to be intermediate in this transition. (**c**) Assembly of eukaryotic histone tetramer. Eukaryotic tetramer resembles that of archeal tetramers

with different DNA topology. HMfB has been shown to cause greater degree of DNA compaction than HMfA. Consistent with this, abundance of these two proteins differ in different life cycle stages (Sandman et al. 1994). Number of histone genes in euryarchaeota lineage goes upto 176 with the exception of *Thermoplasma* having no histone genes. Instead it has HU like proteins. Archeal histones HmfA

and HmfB can form both homodimers and heterodimers. A tetramer of these proteins has been shown to protect 60 bp of DNA indicating single round wrapping of DNA (Pereira et al. 1997).

Collectively, organization of DNA elements with the help of compacting proteins is a common phenomenon for all organisms. In archaebacteria the first step towards the ordered organization of genome with histones could be observed.

1.3.2 Eukaryotic Chromatin

Larger genome and more sophisticated circuit in eukaryotes necessitate ordered and more efficient genome organization. In eukaryotes, linear chromosomes are formed by several levels of DNA compaction. But here also, fundamental unit is nucleosome formed by DNA wrapped around histone proteins. There are four eukaryotic nucleosome core histones namely H2A, H2B, H3 and H4. They are highly conserved and are encoded by different histone genes found in clusters. Eukaryotic histones have a common globular domain and N and C terminal extended tails which are not essential for nucleosome assembly (Fig. 1.2a). These tails interact with regulatory proteins. Eukaryotic histones form exclusive H2A-H2B and H3-H4 heterodimers and not other homodimers. H3 and H2B proteins additionally have five adjacent amino acids that are not found in H4 and archeal histones. Thus H3 and H2B have larger alpha helix $\alpha 1$ and loop L1 than H4 and H2A resulting in asymmetrical H3-H4 and H2A-H2B dimer (Luger et al. 1997).

Crystal structure of nucleosome core particle revealed 146 bp DNA wrapped around the histone octamer core. Histone octamer consists of four histone dimers (H2A-H2B as dimers and H3-H4 as a tetramer) around which DNA is wound 1.65 times. Histone tails extend out beyond the nucleosome. Linker histones do not share the histone fold of canonical core histones. These histones bind near the dyad axis, entry-exit point of DNA in nucleosome thus organizing additional 20 bp resulting in a complex called chromatosome (Hayes et al. 1994). However, exact location of linker histone H1 is yet to be elucidated. During nucleosome assembly, two H3-H4 dimers associate to form a tetramer (Fig. 1.2c). This tetramer recognises nucleosome positioning signal and binds to DNA. The H2A-H2B dimers flank the tetramer from both side followed by DNA wrapping around it (Wolffe 1992; Luger et al. 1997). Interface of two H3 monomers in central H3-H4 tetramer is designated as the dyad axis of symmetry.

1.3.3 Evolution of Eukaryotic Chromatin

Archeal and eukaryal histones share homology both in amino acid sequence and their 3-D structure (Starich et al. 1996; Zhu et al. 1998; Luger and Richmond 1998).

Archeal histones do not contain N and C terminal extensions beyond the histone fold and thus are shorter than eukaryal histones. Eukaryotic histone tails are not required for nucleosome assembly and can be proteolytically chopped without affecting nucleosome structure (Ausio et al. 1989). Tails of H3 and H4 make contacts with adjacent nucleosomes and thus result in tighter packaging. Dimerization properties of histone proteins seem to be conserved to some extent. Hydrophobic interactions among α-helices of the monomers stabilize the dimer structure. Residues of the hydrophobic surface are identical in HMfA and HMfB and thus are capable of forming both homodimers and heterodimers. On the contrary, hydrophobic amino acid residues present in H2A-H2B and H3-H4 corresponding monomers is complementary and not identical explaining their restricted heterodimer formation. Nevertheless, these residues are present at the same location as that of archeal histones. From an evolutionary perspective, transition might have occurred due to mutation in the hydrophobic patch which restrained the formation of homodimer and allowed only heterodimerization.

Discovery of 'doublet histones' in *Methanopyrus kandleri* was the major advancement in understanding evolutionary link between archeal and eukaryal nucleosomes (Slesarev et al. 1998). *Methanopyrus kandleri* histone has duplicated histone folds encoded by single gene. So, unlike other archeal histones, only one type of dimer and tetramer is possible here (Fig. 1.2b). *M. kandleri* histone dimerizes via its C-terminal helices of each N-terminal histone fold in the doublet. The N-terminal histone fold of the dimer structurally correspond to H3 whereas C-terminal to that of H4 (Fahrner et al. 2001). Several residues in doublet histones show deviation from singlet histones (Reviewed in Malik and Henikoff 2003). The N-terminal domains of doublet histones show a conserved change of lysine to methionine as compared to singlet histones. The change remains persistent in eukaryotic H3, emphasizing common constraint in these two classes of dimers.

Phylogenetic studies regarding order of origin of eukaryotic octameric histones is confusing. Nevertheless, tetramer formation is conserved and is observed both with archeal and eukaryal histones. Archeal histones can form tetramers but not the higher forms. Archeal tetramers resemble H3-H4 tetramer of the eukaryotic nucleosomes (Pereira and Reeve 1998) indicating that H3 and H4 have evolved before H2A and H2B. Specifically, comparison between H3 and HMfB sequence reveals identical hydrophobic core in tetramers formed by these proteins (Luger and Richmond 1998). Also, self dimerization of H3 in octamer resembles that of *M. kandleri* doublet histones whereas H2A self dimerization seems unusual. These observations lead to two possibilities of eukaryotic histone appearance: (1) duplication of already divergent pair of histone genes followed by sequence divergence and (2) simultaneous duplication and divergence of one gene. Presence of additional unique five amino acids in α helix of H3 and H2B argues for the former possibility. Appearance of H2A-H2B dimers have led to the octameric form of the nucleosome. The degree of packaging of DNA has increased as octamer is capable of accommodating nearly two turns of DNA when compared to single turn in tetramers. This transition seems to provide selection benefit to the eukaryotes where high degree and rapid condensation/decondensation of chromatin is required.

Centromeric CenH3 containing nucleosomes better known as hemisomes, donot contain conventional octameric histone core as studied in budding yeast and *Drosophila*. In budding yeast, Cse4-H4 tetramer has been reported at the centromere where a nonhistone protein Scm3 is shown to replace histone H2A-H2B dimers (Mizuguchi et al. 2007). In *Drosophila melanogaster* interphase cells, centromeric nucleosomes are composed of one copy each of CenH3, H4, H2A and H2B. These histones form heterotypic tetramers and associate with approximately 120 bp DNA (Dalal et al. 2007). Like archeal nucleosomes, these hemisomes might represent an evolutionary link to ancient form of nucleosomes.

Protozoa and lower eukaryotes like yeast share conserved nucleosome core particle structure with minor differences. Crystal structure reveals slightly destabilized nucleosome core particle in *Saccharomyces cerevisiae* (White et al. 2001). Yeast nucleosomes have shorter repeat length than higher organisms and are more closely spaced (Horz and Zachau 1980). *Saccharomyces cerevisiae* genome contains two genes for each of the histone proteins unlike the histone gene cluster found in higher eukaryotes. Yeast histones are divergent and have sequence differences throughout the protein body including flexible tails (Baxevanis and Landsman 1998). No linker histone H1 gene could be detected in yeast or *Plasmodium*. However, linker histone homolog protein Hho1p has been reported in *Saccharomyces cerevisiae.* The *Hho1p* is not essential as its null mutant is not lethal. Possibly, it does not play any significant role in yeast chromatin organization (Patterton et al. 1998).

Like higher eukaryotes, *Plasmodium falciparum* chromatin is composed of nucleosomal building blocks. However, higher order chromatin organization is lacking probably due to absence of linker histones. *Plasmodium* genome encodes all four canonical histone genes and additional histone variants genes as well (discussed later). Like bacterial chromosomal proteins, *Plasmodium* histones also show life cycle specific expression pattern. Histones are highly expressed in the late trophozoite and schizont stages when DNA synthesis is maximum (Miao et al. 2006).

Histone fold does not seem to be restricted to histone proteins. In eukaryotes, multiple transcription factors like TAFII, PCAF, CBF etc. possess histone folds (Kokubo et al. 1994; Birck et al. 1998; Ogryzko et al. 1998; Ouzounis and Kyrpides 1996). Relative position of DNA binding elements in these histone fold containing proteins differ from that of eukaryotic histones indicating different functions of these proteins. Apart from histones, there are chromatin interacting nonhistone proteins which confer fluidity to the genome. These nonhistone chromatin proteins are not being discussed here due to space constraints.

Chromatin is a nonstatic entity committed to perform multifaceted activities. Conserved structure of histones and their interactions with each other and DNA restrict their functional diversity. Spatial and temporal operations require additional levels of regulation. The chromatin remodelling machineries and covalent modifications of histones are the major players to perform this job. Recently, histone variants have also been shown to modulate various chromatin related phenomena like transcription, silencing, DNA repair, heterochromatinization, meiosis etc. Histone variants harbour minor changes in their primary sequences as compared to canonical histones but result in alteration in chromatin structure (Dryhurst et al. 2004;

Table 1.1 List of histones and histone variants in different organisms

Organisms	Core histones	Core histone variants
Archaea	HMfA, HMfB	–
Yeast	H2A, H2B, H3, H4	H3.3, Cse4, Htz1,H2B1, H2B2
Plasmodium	H2A, H2B, H3, H4	H2Az, H2Bv, H3.3, CenH3
Drosophila	H2A, H2B, H3, H4	H3.3, Cid, H2Av
Mammals	H2A, H2B, H3, H4	H3.1, H3.2, H3.3, CENPA, H2AZ, H2AX, macroH2A, H2A.Bbd, TH2B, H2BFW, SubH2BV, H2BL1, H2BL2

Ramaswamy et al. 2005). Histone variants are encoded by single copy genes located outside the canonical histone genes cluster. Unlike histones, variants genes contain introns and are transcribed as polyadenylated mRNAs. Variants have acquired differential expression pattern contrary to ubiquitous histone expression. Histone variants seem to have evolved from their counterparts with different propensities and through multiple independent events (Thatcher and Gorovsky 1994). Among the four canonical histones, H4 is invariant and most of the H2B variants are involved in spermatogenesis process (Zalensky et al. 2002; Churikov et al. 2004; Aul and Oko 2002; Govin et al. 2007). H2A has a rich family of variants in several different organisms and H3 has a few but functionally important variants. Heterogenity in histone variant propensity is obvious. Basically, only one partner of the allowed heterodimer (H2A-H2B and H3-H4) is varied at a time. It seems only one of the histone partner is selected for variation that will cause least perturbation in nucleosome structure (Reviewed in Malik and Henikoff 2003; Pusarla and Bhargava 2005). Table 1.1 shows the list of histone variants in different organisms.

In eukaryotes, some DNA sequences show high preference for nucleosome assembly which are termed as nucleosome positioning sequences (NPS). It is believed that H3-H4 tetramer recognises NPS and initiates nucleosome assembly (Dong and van Holde 1991; Spangenberg et al. 1998). In archaea too, NPS have been reported. Six or multiple repeats of CTG have been shown to be recognised by archeal histones (Sandman and Reeve 1999).

1.3.4 Evolution of Nucleosome Positioning

It has long been debated whether DNA sequence determines nucleosome deposition or not. After thirty years of research in this field, it has now become clear that DNA sequence does influence nucleosome positioning. DNA wrapped around nucleosomes is far more sharply bent than unstressed DNA which means significant free energy would be needed for the stability of the nucleosomal organization. Certain DNA sequences could reduce the amount of free energy needed by having an inherent bendedness or flexibility. Indeed it has been shown that certain dinucleotides present at the right positions might help in bending. Work by Segal et al. showed that

certain dinucleotides AA/TT/TA exhibited a ~10 bp periodicity; the dinucleotide GC showed the same periodicity, however, GC was out of phase with AA/TT/TA. These nucleosome signatures were obtained by comparing genomic sequences from several organisms, (eg. chicken, yeast, mouse) as well as random *in vitro* synthesized DNA which showed maximum affinity for the histone octamer (Segal et al. 2006).

The nucleosome positioning pattern has been more refined now and has been derived by three independent approaches: analysis of nucleosome DNA sequences, deformational properties of the dinucleotide stacks of nucleosome DNA, and Shannon N-gram extension for genomic sequences (Trifonov 2011b). The pattern derived from all these three approaches were found to be the same when about 160,000 nucleosome DNA sequences from *C. elegans* were analyzed (Gabdank et al. 2009). The nucleosome positioning sequences followed the pattern CGRAAATTTYCG (where R represents purines A or G and Y represents pyrimidines C or T) or YRRRRRYYYYYR in binary form. The CGRAAATTTYCG pattern indicates the positions where certain dinucleotide combinations should be present in the DNA to make the bending energetically less expensive and thus thermodynamically more favourable when wrapped around a histone octamer. The CG dinucleotides reside at the minor grooves which are oriented inwards contacting the surface of the histone octamer whereas the central AT dinucleotides that are five bases away (half-period of DNA), are positioned at the dyad axis of the structural DNA repeat in the minor grooves oriented outwards. Theoretically expressed, the nucleosome positioning pattern reflects the deformational properties of DNA and rules of base stacking interactions which explain why weak bases (W) should be at the minor grooves facing outwards and the strong bases (S) should be at the minor grooves contacting the octamer. The nucleosome positioning pattern finally comes down to ….SSSSWWWWWWSSSSWWWWWW…. sequence for an AT rich eukaryotic genome (S stands for strong dinucleotides CC, GG, GC and CG and W stands for weak dinucleotides AA, TT, AT and TA). Further, the pattern has perfect complementary symmetry due to which the complete identity of the two strands of the DNA duplex can be derived (Trifonov 2011a). This pattern was also found to be completely consistent with the nucleosome DNA patterns of other eukaryotes as established earlier and hence it can be considered to be a consensus nucleosome DNA sequence across eukaryotic species (Rapoport et al. 2011). The actual nucleosomal DNA sequences are not exact matches of the repeating pattern. They are usually rather weak resemblances to the pattern, as an exact match will lead to highly positioned nucleosomes throughout the genome and the information coding capacity of the sequences would be significantly lower. However, the nucleosome DNA sequences form different organisms do show specific dinucleotide sequences at positions matching the nucleosome positioning pattern (Trifonov 2011a).

Recent work on the analysis of DNA sequences in prokaryotes and archaea also showed the periodical patterns of (AAAAATTTTT)n and (GAAAATTTTC)n respectively which are the same as the eukaryotic nucleosome positioning pattern YRRRRRYYYYYR. This study found that although prokaryotes and some species of archaea (Crenarchaea) lack nucleosomes, their sequences do possess the nucleosome positioning code (Rapoport and Trifonov 2011). Archaea that possess

primitive nucleosomes (Euryarchaea) also have been shown to possess the same nucleosome positioning sequence as eukaryotes (Bailey et al. 2000). Some prokaryotic histone like proteins such as HU, H-NS, Fis etc. have also been shown to recognise specific DNA sequences. H-NS binds more preferentially to AAATT sequence (Bouffartigues et al. 2007) whereas Fis binds to AAAWTTT sequence (Hengen et al. 1997) which is a part of the nucleosome positioning motif. It has now been proposed that although prokaryotes do not have DNA folded into nucleosomes, they do have nucleosome positioning sequences which appear to have evolved before the prokaryote-eukaryote separation (Rapoport and Trifonov 2011).

Nucleosomal arrangement of genome not only serves the packaging purpose but it encompasses functional significance as well. Nucleosomes inherently possess repressive properties, which the genome cannot withstand throughout the cell cycle stages. Genome has to follow ordered opening and closing for proper functioning. Several factors like remodelers, DNA and histone modifying enzymes and histone chaperones effect fluidity and dynamicity of chromatin. There is a very precise and complicated interplay among these effectors to fine tune the life processes which is not yet fully understood.

1.3.5 Evolution of Lysine Acetyltransferases

Acetylation, the most widely studied posttranslational modification of histones and nonhistone proteins is catalyzed by different classes of lysine (K)acetyltransferases (KATs). The type A KATs are nuclear whereas type B KATs are cytoplasmic. Lysine acetylation is a highly conserved phenomenon observed from archaea, protozoan to humans. The Haloarchaeal species *H. Volcanii* genome contains GCN5 family acetyltransferase (Pat1, Pat2 and Elp3) (Altman-Price and Mevarech 2009). The Alba family of proteins in *S. solfataricus* also gets acetylated by *Salmonella* protein acetyltransferase (PAT) at lysine 16 (Bell and Grogan 2002; Eichler et al. 2005). While some KATs like Elp3 are present in both archaea and eukaryotes suggesting the occurrence of these enzymes early in evolution (Reeve 2003), some enzymes are restricted to the eukaryotes, like the MYST family of KATs and p300/CBP family of KATs. p55, a type A nuclear KAT is present in the protozoan *Tetrahymena*. This protein is surprisingly a homolog of GCN5 and contains a highly conserved bromodomain (Brownell et al. 1996). Apart from archaea and protozoans, two classes of KATs are found in malarial parasites. The *P. falciparum* genome codes for a GCN5 (PfGCN5) and pfMYST (shares homology with yeast Esa1) which is essential for malarial infectivity (Cui and Miao 2010) (Table 1.2).

In eukaryotes, KATs are present in functionally distinct protein complexes. The yeast GCN5 is found in two high molecular mass complexes, SAGA and Ada. The SAGA complex is known to activate transcription and acetylates nucleosomal histone H3 and H2B (Eberharter et al. 1999; Grant et al. 1997). Esa1 in yeast is essential for growth (Smith et al. 1998) whereas Rtt109 is essential for acetylation

Table 1.2 Evolution of different Lysine acetyltransferases from archaea to humans

Organism	PCAF/GCN5	CBP/p300	MYST
Archaebacteria	*H. volcanii* contains three protein acetyltransferases (Pat1, Pat2 and Elp3) belonging to the GCN5 family of acetyltransferases	–	–
Protozoa	p55 (Homolog of GCN5)	–	–
Plasmodium	PfGCN5	–	PfMYST
Yeast	yGCN5	Rtt109 (Homolog of p300)	Sas3 and Esa1
Drosophila	GCN5 containing KAT complexes	dCBP (No p300 is identified)	dMOF
Human	PCAF/GCN5	p300/CBP	Tip60 and MOZ, HBO1 and MORF

of histone H3K56. *Drosophila* lacks p300 homolog but CBP is present and deletion of dCBP results in cell cycle arrest and stalled DNA replication (Smolik and Jones 2007). GCN5 in *Drosophila* is present in two different complexes STAGA and ATAC and is involved in acetylation of histone H3 and H4 (Kusch et al. 2003; Martinez et al. 1998). Chameau is a MYST family KAT present in *Drosophila* required for maintenance of Hox gene silencing (Grienenberger et al. 2002). In mammals, p300/CBP has diverse function including acetyltransferase activity, E3 ubiquitin ligase and transcriptional coactivation. PCAF/GCN5 mediated acetylation of histones and non histone proteins are essential for transcriptional activation and also muscle differentiation. Tip60 (homolog of Esa1), a MYST family KAT (in mammals) mediated acetylation is essential for DNA damage response. Remarkably, among all the KATs, the Esa1/Tip60 seems to be an essential gene. Evolutionarily, this KAT is conserved from protozoans to human. However, the p300/CBP family of KAT seems to be more mammalian specific. Deletion of either of them is not lethal but knockout of both is life threatening (Yao et al. 1998; Kasper et al. 2006).

1.3.6 Evolution of ATP Dependent Chromatin Remodelers

Apart from chromatin modification systems, the ATP dependent chromatin remodelling machineries also significantly contribute to chromatin dynamics by mobilising the nucleosomes on DNA based on epigenetic signals. Chromatin remodelers are classified into four families: SWI/SNF (switching defective/sucrose nonfermenting), INO80 (inositol requiring 80), ISWI (imitation switch) and CHD (chromodomain, helicase, DNA binding) family (Clapier and Cairns 2009). Among these complexes the INO80 family of chromatin remodelers, seems to be highly conserved and

functionally significant. Due to space constraint, we will discuss only about the INO80 family of chromatin remodelers in this section. INO80 is a class of chromatin remodelers comprising of INO80 and SWR1 complexes. These complexes remodel chromatin by interacting with the nucleosome and perform nucleosome sliding along the DNA or exchange histones within the nucleosome (Bao and Shen 2011). The INO80 complex also recruits various regulatory factors to the nucleosome. Homologs and orthologs of this family are present in yeast, *Drosophila*, plants and mammals. The ATPase subunit of INO80 possesses the maximum homology among different organisms. INO80 and SWR1 complexes are composed of 15 and 14 subunits respectively with approximately1.2–1.5 MDa mass of each. The various members of this family of proteins are Ino80 (INOsitol requiring), Rvb1, Rvb2, Arp4 (Actin-related protein 4), Arp5, Arp8, actin, Nhp10 (Non-histone protein 10), Ies1 (Ino Eighty Subunit 1), Taf14 (TATA-binding protein-associated factor) Ies2, Ies3, Ies4, Ies5 and Ies6. One distinguished characteristic of INO80 subfamily is the presence of RuvB-like helicases in the complexes. RuvB-like helicases are especially present in bacteria as DNA repair factor. Yeast INO80 ATPase domain bears significant resemblances with yeast Snf2/Swi2 family of DNA-dependent ATPases. One additional feature in INO80 ATPase domain is the presence of a spacer inside the conserved ATPase domain (Ebbert et al. 1999). TELY motif present in the N-terminal domain of INO80 is conserved in yeast, *Drosophila* and human and is known to be the interacting residue for actin, Arp4, Arp8 and Taf14/Anc1 (Shen et al. 2000, 2003). The SWR1 complex is composed of 14 members and shares homology with INO80 subunits (Fig. 1.3a). The members of SWR1 complex are Swr1, Swc2/Vp372, Swc3, Swc4/Eaf2/God1, Swc5/Aor1, Swc6/Vps71, Swc7, Yaf9 (yeast homolog of the human leukemogenic protein AF9), Bdf1 (bromodomain Factor), Act1/actin, Arp4, Arp6, Rvb1 and Rvb2 (Mizuguchi et al. 2004; Wu et al. 2005; Bao and Shen 2007). Actin, Arp4, Rvb1 and Rvb2 are the common shared subunits between INO80 and SWR1 complexes. Rvb1 and Rvb2 proteins are class of AAA+ ATPases (ATPases associated with a variety of cellular activities) and are related closely to the Holliday junction-resolving RUVB proteins present in bacteria (Shen et al. 2000). Rvb1 and Rvb2 are crucial components of the INO80 and SWR1 remodeling complexes, Tip60 acetyltransferase complex (Ikura et al. 2000) and c-MYC complex. The Ies2 and Ies6 proteins of INO80 complex are also highly conserved from yeast to mammals (Fig. 1.3b) including humans and have distinct roles in transcription regulation (Cai et al. 2007; Shen et al. 2003). Recent report suggests that Iec1 is another novel component of INO80 complex and bears sequence similarity to the *Drosophila* and human counterparts. Iec1 is essential for transcriptional regulation and with other components of INO80 complex (arp8, ies6, and ies2) plays crucial roles in DNA damage repair (Hogan et al. 2010).

The *Drosophila* ortholog of INO80 is Pho-dINO80 and that of NuA4 complex is Tip60. *Drosophila* SWR1 complex comprises of BAP55 and Arp. These proteins are also present in BAP and PBAP complexes. The SWR1 complexes functions in the exchange of H2A variants and is conserved throughout evolution. *Drosophila* Tip60 complex helps in the exchange of H2A for H2Av, which is a variant of H2A

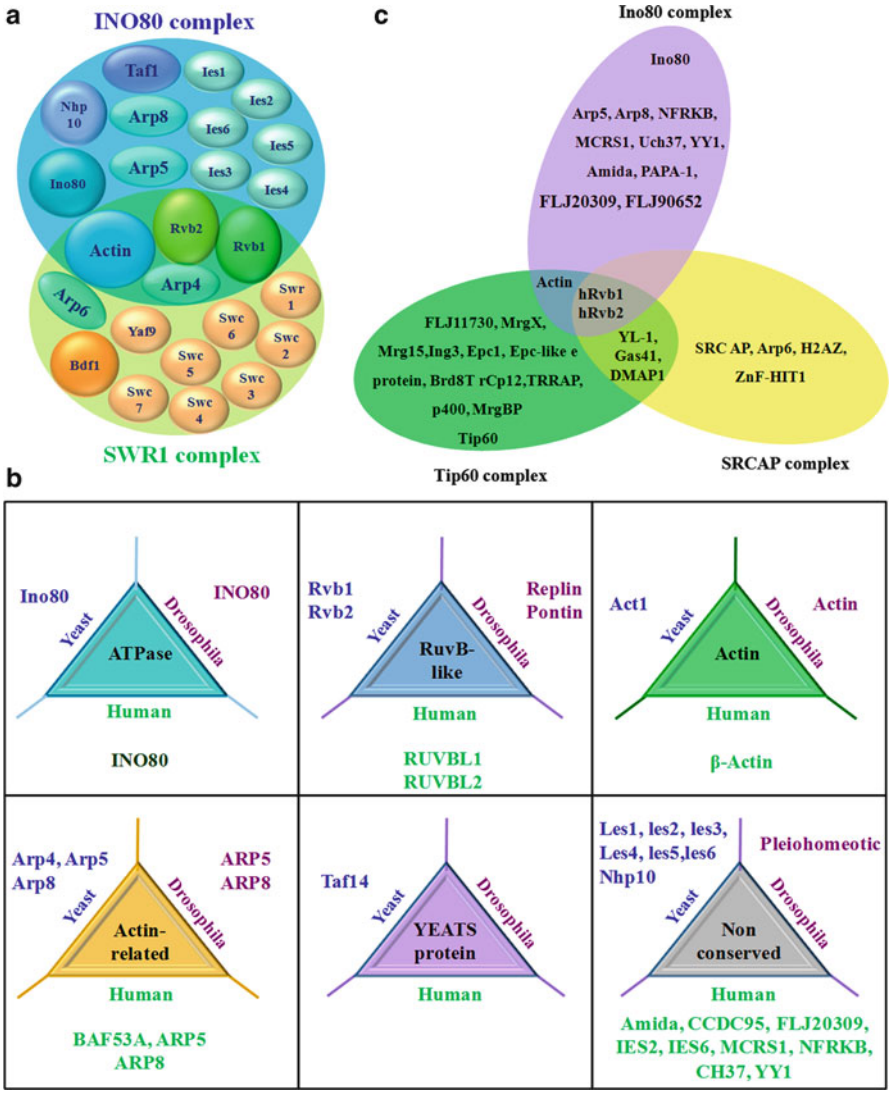

Fig. 1.3 (**a**) Components of yeast INO80 (inositol requiring 80) and SWR1 complexes. The structural-functional homologs are common among the two complexes. (**b**) Homologous factors of INO80 complex among yeast (*Saccharomyces cerevisiae*), *Drosophila* and humans. Each *triangle* represents different subunit types of INO80 complex. (**c**) Protein subunits of Ino80, Tip60 and SRCAP complexes in humans

having resemblances with both H2AZ and H2AX (Kusch et al. 2004). SRCAP complex, the mammalian homologue of SWR1 also catalyzes the exchange of H2A to H2AZ (Ruhl et al. 2006; Wong et al. 2007). Based on a number of shared subunits, *Drosophila* Tip60 (dTip60) is similar to yeast SWR1, but also contains additional

seven subunits including a histone acetyltransferase subunit (Tip60). dTip60 is presumed to be an evolutionary product of combined SWR1 and NuA4 complexes in yeast (Hargreaves and Crabtree 2011). In humans the related Tip60 complex is Tip60/ TRRAP complex which possesses three distinct enzymatic activities: histone H4/H2A acetyltransferase, ATP-dependent H2AZ-H2B histone dimer exchange, and DNA helicase. Tip60/TRRAP complex is considered to be the merge of NuA4 and SWR1 complexes through evolution by fusion of Swr1 and Eaf1 (Auger et al. 2008). The major components of human Tip60/TRRAP complex are Swr1-related ATPase, p400, and Tip60. Arp4 and/or actin are similar throughout evolution and are common in yeast, *Drosophila* and humans (Fig. 1.3c). The Swr1 homologue in *Drosophila* is Domino and SRCAP and p400 in humans (Cai et al. 2005). The human homologues of yeast Rvb1 and Rvb2 are RUVBL1 and RUVBL2 while they are named Reptin and Pontin in *Drosophila*. Tip60 in all the organisms are recruited to DNA double strand breaks presumably by an Arp4 dependent mechanism. Subsequently in yeast, NuA4 acetylates histone H4 at break points (Downs et al. 2004), in *Drosophila* Tip60 acetylates phosphorylated H2Av at DNA break points and catalyses exchange with H2A (Kusch et al. 2004). In humans, Tip60 acetylates γH2AX and upon DNA damage facilitates its removal (Ikura et al. 2007). In addition to the INO80 ATPase and other core subunits, human INO80 complex contains Gli-Kruppel zinc finger transcription factor Yin-Yang 1 (YY1), the deubiquitylating enzyme Uch37 and nuclear factor related to κB (NFRKB). *Drosophila melanogaster* polycomb group protein Pleiohomeotic (Pho) is the ortholog of human YY1 and also contains Uch37 and NFRKB (Conaway and Conaway 2009). Significant role of INO80 and SWR1 has been identified in regulation of telomere structure (Yu et al. 2007). Thus, INO80 family of chromatin remodelers function in various cellular processes like DNA damage repair, telomere stability and cell cycle checkpoint. One of the major features of this class of complexes is the recognition of histone variants and recruitment to various structures like DSB sites, holliday junctions and telomeres etc. This function of the complexes is facilitated by the presence of the unique RuvB like proteins and split ATPase domains in various organisms.

Apart from chromatin modifications and remodeling, histone chaperones also significantly contribute in the chromatin dynamics and thereby the operation of epigenetic phenomenon.

1.3.7 Evolution of Histone Chaperones

Histone chaperones are histone interacting proteins that are involved in all types of histone metabolism such as histone storage, transport and assembly and disassembly of histones on chromatin. Due to their involvement in nucleosome assembly and disassembly, histone chaperones have been found to play important roles in essential processes such as replication, transcription and repair. Several histone chaperones have evolved from yeast to mammals and exhibit conservation of

sequence and function. Due to space constraint, we will only be discussing about the Nucleoplasmin family.

Nucleoplasmin was the first histone chaperone to be identified. It was identified as a factor in *Xenopus* egg extracts that binds to histones and assembles them onto the DNA in a cell free nucleosome assembly system (Laskey et al. 1978). Since then, many other homologs of Nucleoplasmin have been identified and they comprise the Nucleoplasmin/Nucleophosmin (NPM) family of histone chaperones. Nucleoplasmin homologs are present throughout the animal kingdom but are not found in lower unicellular organisms such as yeast (Frehlick et al. 2007). Based on the protein sequences they are divided into four groups, NPM1, NPM2, NPM3 and invertebrate NPM like protein (Frehlick et al. 2007).

The Nucleoplasmin family members are characterized by the presence of a conserved N-terminal domain (also called the core domain) and a highly divergent C-terminal domain. The N-terminal domain is responsible for oligomerization of the subunits as well as for histone chaperone activity in some members. It contains an acidic stretch A1 which is important for sperm decondensation activity of the members (Salvany et al. 2004). However, human NPM1 differs from the other family members in the fact that histone chaperone activity resides in its C-terminal domain (Swaminathan et al. 2005) and the N-terminal domain has a rudimentary A1 consisting of two amino acids which might not be functional. Moreover, human, mouse and rat NPM2 also donot possess the A1 acidic stretch (Frehlick et al. 2007). The C-terminal domain contains one or more acidic tracts (A2 and/or A3) which are responsible for histone binding (Swaminathan et al. 2005). Nucleoplasmin family members possess a classic bipartite nuclear localization signal $KRX_{10}KKK$ where X is any amino acid (Dingwall et al. 1987). Apart from these domains, some members like NPM1 have additional Nuclear Export Signal (NES), Nucleolar Localization Signal (NoLS) and a nucleic acid binding domain that binds to RNA and is required for rRNA cleavage (Hingorani et al. 2000).

The earliest occurrence of the Nucleoplasmin family has been found in the invertebrates and they are known as Nucleoplasmin like proteins (NLPs). The best characterized among them is the *Drosophila* NLP (dNLP) (Ito et al. 1996). dNLP has been shown to bind to core histones and exhibits nucleosome assembly activity but additionally requires ATP unlike other members and the help of atleast one other assembly activity. Moreover, dNLP was not able to promote sperm decondensation unlike other members of the family (Ito et al. 1996). NPM1 (Nucleophosmin) or NO38 in *Xenopus* is a nucleolar protein. The human homolog Nucleophosmin was first identified as a nucleolar phosphoprotein (Kang et al. 1974; Prestayko et al. 1974) and since then has been extensively studied. The histone chaperone activity of NPM1 was demonstrated later (Okuwaki et al. 2001) where it was shown to bind both H2A-H2B dimer as well as the H3-H4 tetramer without any preference (Swaminathan et al. 2005). Unlike its human homolog, the *Xenopus* NPM1 (NO38) shows a preference towards H3-H4 tetramer (Namboodiri et al. 2004). NPM1 also binds to linker histone H1 and possesses the linker histone chaperone activity (Gadad et al. 2011). NPM1 has been implicated in many other cellular processes

which are reviewed in (Grisendi et al. 2006; Okuwaki 2008). NPM2 or Nucleoplasmin of *Xenopus* is the founder member of the family and has been extensively studied (Laskey et al. 1978). NPM2 is found only in eggs and oocytes (Mills et al. 1980) and binds to H2A-H2B dimer preferentially (Dutta et al. 2001). NPM2 is involved in storage of H2A-H2B dimer in the oocytes and sperm chromatin decondensation following fertilization. It displaces the sperm basic proteins and deposits H2A-H2B dimer on to the paternal chromatin (Frehlick et al. 2007). NPM3 was identified later (MacArthur and Shackleford 1997) and was shown to regulate the ribosome biogenesis (Huang et al. 2005) and histone chaperone activity of NPM1 (Gadad et al. 2010). NPM3 binds to all the core histones, yet, in *in vitro* supercoiling assays, it did not show any nucleosome assembly (Gadad et al. 2010). However, it may be involved in the regulation of sperm chromatin decondensation (McLay and Clarke 2003).

The crystal structures of the N-terminal domain of *Xenopus* Nucleoplasmin, NO38, human NPM1, NPM2 and dNLP have been solved and all of them were shown to form a pentameric structure. However, the functional form of all the members is a decamer formed by head-to-head association of two pentamers (Dutta et al. 2001; Namboodiri et al. 2003, 2004; Lee et al. 2007; Platonova et al. 2011). Biochemical and modeling studies suggest that each decamer can bind to five histone octamers (Dutta et al. 2001). Further, in case of human NPM2, it was shown that it forms decamers when it binds to both H3-H4 tetramer and H2A-H2B dimer simultaneously. However, when it binds to H2A-H2B dimer alone, it remains as a pentamer (Platonova et al. 2011).

NPM family has been shown to undergo functional evolution and diversification (Eirín-López et al. 2006). Phylogenetic analyses on the NPM family have revealed that the NPM family members of a single type (such as NPM1, NPM2, NPM3 or invertebrate NLP) cluster together by type rather than by species in the phylogenetic tree. The NPM1 and NPM-like lineage showed to have a monophyletic origin whereas NPM2 and NPM3 showed a polyphyletic origin having evolved independently in mammals as compared to the other vertebrates. The NPM-like lineage from invertebrates was found to be phylogenetically closer to NPM1. NPM sequences were found to have diverged extensively through silent substitutions indicating the presence of selection acting on specific residues especially glutamate and aspartate in the acidic tracts (Eirín-López et al. 2006). The acidic residues in the acidic tracts are important for their interactions with histones and the chaperone activity and hence, are maintained by selection. Even histones are known to evolve similarly by a selection process by silent substitutions operating at the nucleotide level in order to conserve the basic residues (Frehlick et al. 2007).

1.3.8 Epigenetics and Differentiation

As discussed in earlier sections, genome organising and modifying machinery have gone through systematic development through the course of evolution. This sequence

of events is well-documented in the phenomenon of differentiation, which is associated with global changes in genome organisation and is characterised by fluctuations in gene expression. The role of chromatin modifying machinery in genome organisation, in modulating the balance between "stemness" and differentiation in multiple lineages has been studied extensively. This section will briefly discuss the conserved molecular role of the biochemical phenomena of histone modifications, and DNA methylation during differentiation, taking the neural system as an example.

1.3.8.1 Epigenetics and Neural Differentiation

Till few decades ago, neurons were not known to regenerate, but the scenario changed with the discovery of neural stem cells (NSCs), which are primarily generated in the subgranular zone (SGZ) of the hippocampal dentate gyrus, and the subventricular zone (SVZ). The NSCs can differentiate into three principal types of cells- Neurons, Astrocytes and Oligodendrocytes. The role of epigenetic modulators in the process of neural differentiation has been well-elucidated over the past two decades. The switch from neurogenesis to astrogliogenesis during mid to late gestation period is primarily modulated by the JAK-STAT pathway controlled by DNMT1 (Fan et al. 2001, 2005; Namihira et al. 2009). The methylation of STAT3-binding element (CpG) in the GFAP promoter occurs during neurogenesis (Bonni et al. 1997) and astrogliogenesis is accompanied by demethylation of these elements in the GFAP promoter, along with other astrocyte-specific gene promoters (Shimozaki et al. 2005).

The histone deacetylases 1 and 2 (HDAC 1 and 2) modulate neural differentiation (Montgomery et al. 2009), and are associated with both induction as well as regulation of differentiation process. While it was observed that treatment of NSCs with VPA, an HDAC inhibitor, induced neuronal differentiation (Hsieh et al. 2004), HDAC activity is required for oligodendrocyte differentiation, and during myelination (Marin-Husstege et al. 2002; Shen et al. 2005). However, paradoxically HDACs are known to be primarily associated with gene repression, thus the mechanism of HDAC action in the differentiation process is yet to be understood. The involvement of specific miRNA which are regulated by HDACs cannot be ruled out. Recently, the role of miRNA in the neural differentiation has indeed been implicated. In the process of neural specification, miR-124, a brain specific miRNA (Lagos-Quintana et al. 2002), is upregulated during differentiation of progenitor cells to mature neurons (Deo et al. 2006). This miRNA is required for silencing of non-neural genes in neural cells (Lim et al. 2005; Conaco et al. 2006). The miR-124 along with miR-9, regulates the STAT3 pathway mediated glial-specific gene expression, by inhibiting STAT3 phosphorylation, and hence, astrocyte differentiation (Krichevsky et al. 2006), indicating multiple roles of the miRNA in regulation of gene expression and lineage specification.

1.3.8.2 Epigenetic Control: Linking Invertebrate and Mammalian Differentiation

Chromatin in stem cells is distinctly different from differentiated cells. Though the gross structure and organ systems vary to a large extent between species, the epigenetic factors regulating development seem to show significant conservation with diversification. The comparative analysis of the chromatin signatures of mammalian embryonic stem cells shows a high degree of conservation. The characteristic open, pliable chromatin was observed in early embryos, with DNA methylation-dependent transcription regulation in the later stages during organogenesis and differentiation (Bogdanovic et al. 2011). Histone modifications are seen to be involved both in transcriptional regulation and chromatin organization during development, across species. As seen with human embryonic stem cell (hESC) differentiation, there is a characteristic shift from the abundance of transcriptionally active to repressive marks during differentiation in *Xenopus* (Schneider et al. 2011). The overall role of histone acetylation and HDAC activity in influencing *Xenopus* development has been studied (Shechter et al. 2009; Almouzni et al. 1994). Specifically, in zebrafish, HDAC1 plays the role of a transcriptional activator in neurogenesis and CNS development (Harrison et al. 2011). H3 acetylation in *Drosophila*, shows a stage-specific increase during gastrulation, associated with high levels of transcription (Harisanova and Ralchev 1986). Similarly, histone methylation patterns (both lysine and arginine methylation) in the three organisms have been shown to be associated with differentiation (Peng et al. 2009; Fujii et al. 2011; Tao et al. 2011; Cakouros et al. 2008; Villar-Garea and Imhof 2008). However, histone modification patterns differ among organisms, as shown in the recent study comparing murine ES cells and *Xenopus* pluripotent cells (Schneider et al. 2011). Although, both murine and *Xenopus* pluripotent cells share high H3K4me2/me3 levels, the H3K27me3 mark is more than 100-fold enriched in murine ES cells as compared to *Xenopus* blastulae. Also, combinatorial tri and higher methylated states of H3 K27 and H3 K36 were significantly more enriched in murine than in *Xenopus* pluripotent cells. The functional significance of these organism-specific epigenetic modifications is yet to be elucidated.

As in mammals, DNMTs have been observed to regulate gene expression in a stage-specific manner in other vertebrates as well. For instance, DNMT1 depletion in *Xenopus* is seen to lead to premature differentiation and apoptosis associated with development (Stancheva and Meehan 2000; Stancheva et al. 2001) and DNA hypomethylation is observed during differentiation (Talwar et al. 1984). Similarly, DNMTs, in zebrafish have been shown to be essential in regulating various developmental pathways, including neurogenesis (Rai et al. 2006), pancreatic beta cell differentiation (Anderson et al. 2009), liver, retina and lens development (Rai et al. 2007; Tittle et al. 2011). Significantly, studies during *Xenopus* development have led to the hypothesis that DNMT1 have other functions than solely DNA methylation itself (Hashimoto et al. 2003).

The information summarized above suggests the conserved nature of chromatin modifying machinery, and its role in differentiation. Notably, HDACs, DNA methylation and H3 methylation play essential roles during development and regulate lineage-specific differentiation. The presence of the global open chromatin structure, followed by large scale chromatin organization and remodeling, associated with changes in DNA methylation and histone modification can hence be considered a phenomenon conserved over the classes, ranging from invertebrates to vertebrates.

1.3.9 Perspective

With the increasing complexity of genome structure and function the organizational and modification or remodelling machineries of chromatin has also achieved inconspicuous diversity during the course of evolution. Nevertheless, overall pathway of their development could be traced significantly. In the light of availability of whole genome databases of DNA sequence and epigenetic modifications, our understanding of this pathway will soon be clearer. Collectively, these informations should be highly useful not only to perceive the evolutionary process but also to understand disease biology.

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Chapter 2
Secondary Structures of the Core Histone N-terminal Tails: Their Role in Regulating Chromatin Structure

Louis L. du Preez and Hugh-G. Patterton

Abstract The core histone N-terminal tails dissociate from their binding positions in nucleosomes at moderate salt concentrations, and appear unstructured in the crystal. This suggested that the tails contributed minimally to chromatin structure. However, *in vitro* studies have shown that the tails were involved in a range of intra- and inter-nucleosomal as well as inter-fibre contacts. The H4 tail, which is essential for chromatin compaction, was shown to contact an adjacent nucleosome in the crystal. Acetylation of H4K16 was shown to abolish the ability of a nucleosome array to fold into a 30 nm fibre. The application of secondary structure prediction software has suggested the presence of extended structured regions in the histone tails. Molecular Dynamics studies have further shown that sections of the H3 and H4 tails assumed α-helical and β-strand content that was enhanced by the presence of DNA, and that post-translational modifications of the tails had a major impact on these structures. Circular dichroism and NMR showed that the H3 and H4 tails exhibited significant α-helical content, that was increased by acetylation of the tail. There is thus strong evidence, both from biophysical and from computational approaches, that the core histones tails, particularly that of H3 and H4, are structured, and that these structures are influenced by post-translational modifications. This chapter reviews studies on the position, binding sites and secondary structures of the core histone tails, and discusses the possible role of the histone tail structures in the regulation of chromatin organization, and its impact on human disease.

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2.1 Introduction

2.1.1 The Need for DNA Packaging

The total length of the DNA in a single diploid human cell is approximately 2 m, a length that must fit into a cell nucleus that is roughly 10 μm in diameter. To accomplish this, the DNA is packaged into arrays of nucleosomes. Each nucleosome is formed by spooling about 168 bp of DNA in two negative superhelical turns onto a histone octamer, which is composed of two copies of each of the core histones H2A, H2B, H3 and H4. A fifth histone, linker histone H1, binds to the outside of the structure, close to the point of DNA entry and exit. The H1 causes partial charge neutralisation of the linker DNA, which connects adjacent nucleosomes in the array (Van Holde 1989).

This array of nucleosomes is further condensed into a 30 nm fibre, which is composed of a helical arrangement of nucleosomes. No definitive structural detail is currently available on the 30 nm fibre. The 30 nm fibre undergoes additional levels of folding to form higher order structures, culminating in the condensed structures observed electron microscopically in the metaphase chromosome (Woodcock and Dimitrov 2001).

Although the packaging of DNA into chromatin solves the problem of fitting an extended, poly-anionic, linear polymer into the confined space of a eukaryotic nucleus, a significant problem is introduced in that the DNA molecule also becomes masked from most of the proteins and enzymes that must interact with it as part of its genetic function. Thus, to allow access to the DNA molecule, eukaryotic cells have evolved intricate mechanisms whereby the chromatin is locally and reversibly decondensed. Mechanisms involved in this local decondensation include the structural perturbation of chromatin structures by ATP-dependent chromatin remodelling enzymes, the deposition of different histone isotypes, and the reversible chemical modification of the "histone tails".

2.1.2 Histone Tails: More Than Just Fashionable

The histone tails are seemingly unstructured extensions of the core histones beyond the central histone fold domains (Arents et al. 1991; Luger et al. 1997), and contribute approximately 38% of the core histone mass to the histone octamer (Fig. 2.1). The chemical modification of the histone was first observed by Phillips (1961, 1963) and by Murray (1964). The Mirsky group subsequently showed that acetylation of histones facilitated synthesis of RNA in cell-free extracts (Allfrey et al. 1964). These initial observations defined the beginning of a field that has become known as *Epigenetics* and its high-throughput application, *Epigenomics*. A substantial scientific literature has since developed describing an extensive range of modifications (Fig. 2.1) and the function of these modifications [see Kouzarides (2007) and Kundu (2007) for reviews].

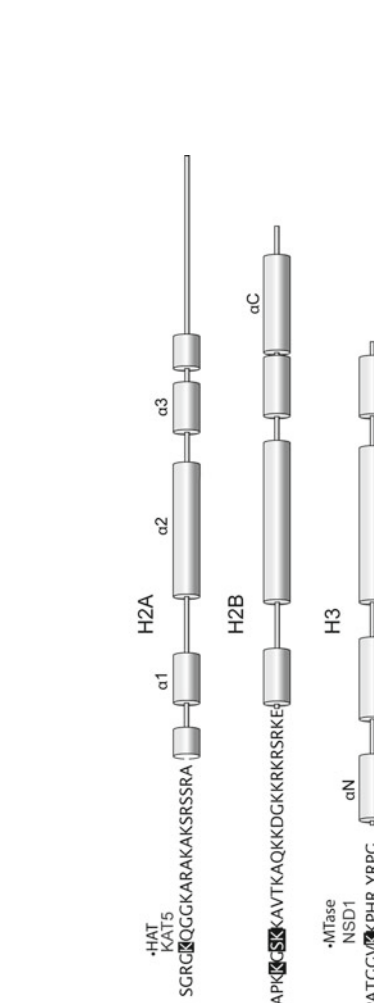

Fig. 2.1 The sites of epigenetic modification in the core histone tails. The core histone tail sequences are shown, as well as the central histone folds with the additional α-N and α-C helices of H3 and H2B. The individual residues that are sites of epigenetic modification are indicated. Human enzymes in the UniProt database (Apweiler et al. 2004) that were annotated with gene ontology terms indicating specificity towards each of these different residues, are identified (http://www.uniprot.org; accessed January 2011)

Early indications were that modified residues served as molecular beacons for the recruitment of specific proteins to such flagged areas of the genome (Strahl and Allis 2000). For instance, it was shown that the heterochromatin-associated protein HP1 was recruited to regions marked for transcriptional silencing by tri-methylated K9 of histone H3 (Bannister et al. 2001). Regulatory effects of one modification on another modification in the same tail or in the tail of a different histone were also discovered, termed *cis*-tail and *trans*-tail pathways, respectively. For instance, phosphorylation of S10 of H3 was shown to inhibit demethylation of the mono- and di-methylated K4, thus maintaining an "active" epigenetic signal (Forneris et al. 2005). Ubiquitination of K123 of histone H2B required a sequence motif in the H2A tail, which is in close proximity to H2B K123 in the nucleosome, and may be involved in the recruitment of the ubiquitination machinery (Zheng et al. 2010). Ubiquitination of H2B K123, in turn, was required for recruitment of the methyltransferase complexes for the subsequent methylation of H3 K4 by Set1 (Dover et al. 2002) and of H3 K79 by Dot1 (Briggs et al. 2002), marks associated with transcriptional activation. This system of molecular flags and interdependencies is known as the histone code, proposing that histone modification represent a template for direct "read-out" by other proteins who then perform specific chromatin-associated functions (Strahl and Allis 2000).

2.1.3 Histone Tails: Beyond the Histone Code

Although there are many instances where this histone code model is an accurate description of biochemical functions *in vivo*, instances were also observed where histone tail modifications represented more than simple molecular beacons. The most striking observation involved K16 of histone H4. *In vitro* data showed that deacetylation of K16 was required for full compaction of chromatin into a condensed fibre in the presence of a linker histone (Robinson et al. 2008). In the absence of H1, acetylation of H4 K16 was also shown to inhibit formation of a condensed structure in a reconstituted nucleosome array, although the relationship of this array to the canonical 30 nm fibre was not determined (Shogren-Knaak et al. 2006; Allahverdi et al. 2011). This represented an example where a histone tail modification had a significant effect on chromatin structure, but was not involved in the recruitment of any protein to accomplish the structural effect in an *in vitro* system composed of purified and defined components. A genome-wide gene expression analysis also demonstrated a redundant, cumulative effect for mutations of K5, K8 and K12 of histone H4 to arginine, designed to mimic the unacetylated state of lysine. The H4 K16R mutation, on the other hand, had a transcriptional effect that was independent of the state of K5, K8 and/or K12, suggesting that acetylation played a fundamentally different functional role in these two groups of residues (Dion et al. 2005). Also, unlike K5 and K12 of H4, which showed a strong correlation between acetylation state and gene expression, there was little correspondence between the acetylation

state of H4 K16 in nucleosomes adjacent to the transcription start site, and the average transcriptional activity of genes (Liu et al. 2005).

Biochemical studies have shown that some chemical modifications of amino acid residues in peptides caused significant changes in the secondary structure of the peptides (Wang et al. 2001). However, very little attention has been given to the possible effect of epigenetic modifications on the secondary structures of the histone tails, and the impact this may have on the association of the tails in chromatin. The fact that the histone tails appeared unstructured in X-ray crystallographic studies, most likely due to the dissociation of the tails from binding sites under conditions of moderate salt (Walker 1984; Luger et al. 1997), may have contributed to an impression that they were structurally unimportant. However, the finding that deacetylation of H4 K16 was required for full compaction of the chromatin fibre (Shogren-Knaak et al. 2006; Robinson et al. 2008; Allahverdi et al. 2011), renewed interest in a direct structural role of the histone tails in chromatin. In this Chapter we review the literature on the effect of amino acid residue modifications on the secondary structures of peptides including histone tails, citing biophysical, biochemical and *in silico* computational studies. We finally discuss how this may impact on chromatin structure and the epigenetic basis of human disease.

2.2 Chromatin Structure

2.2.1 A Model for the 30 nm Fibre

A series of images recorded of chromatin in the presence of a linker histone at increasing salt concentrations showed the systematic compaction of the chromatin through successively more condensed structures, reaching a fibre of approximately 30 nm diameter as a compaction limit (Thoma et al. 1979). This most condensed state of packaging of the nucleosomes relative to each other was termed the "30 nm fibre", which may undergo additional levels of folding into higher-order structures and helices (Woodcock and Dimitrov 2001).

Despite significant effort spanning many decades, there is still no agreement on the exact structural arrangement of nucleosomes in the 30 nm fibre. One model proposed a continuation of the folding of a linear array of nucleosomes into a contact helix or solenoid, where each neighbour in the solenoid was also adjacent in the linear array (Finch and Klug 1976). An alternative model suggested that the fibre was assembled in a manner that placed neighbouring nucleosomes consecutively on opposite sides of the fibre axis, to form a two-start helix, with the linker DNA running through the fibre centre (Worcel et al. 1981; Woodcock et al. 1984; Williams et al. 1986). This latter model has received strong experimental support from cross-linking (Dorigo et al. 2004) and X-ray crystallographic studies (Schalch et al. 2005). Many variations of these two central proposals exist, mainly based on the connectivity between nucleosomes in the fibre (Daban and Bermudez 1998; Robinson et al. 2006).

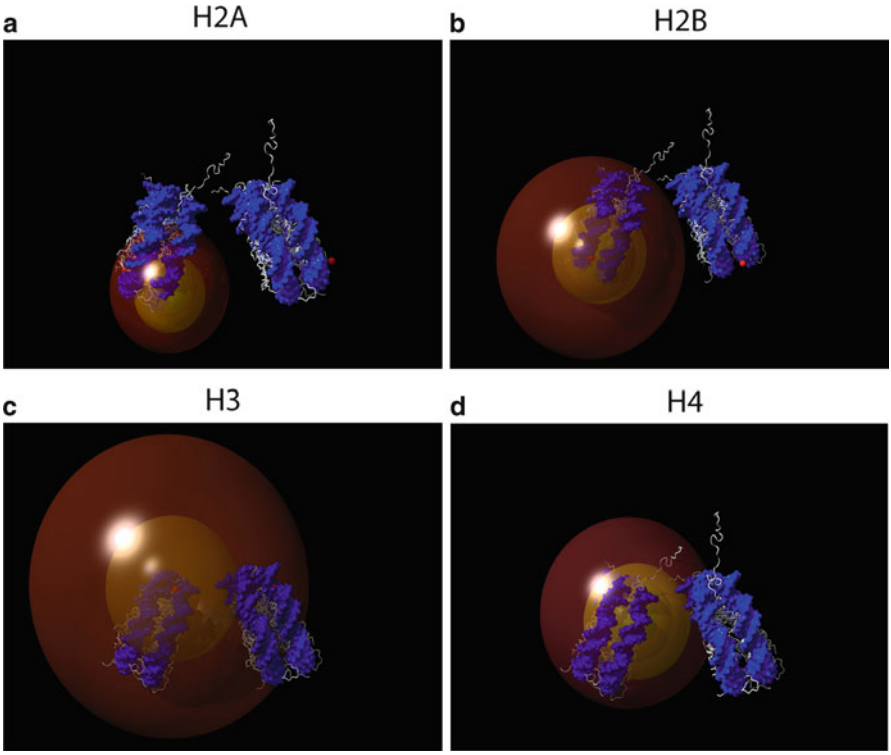

Fig. 2.2 Reach of the N-terminal core histone tails in chromatin. The reach of each of the N-terminal tails of the core histones (**a**) H2A, (**b**) H2B, (**c**) H3 and (**d**) H4 is shown. The volume that can be swept out by each tail is represented by a *sphere centred* on the defined start of each tail (Luger et al. 1997), with the tail maximally extended (3.3 Å per residue) or with the full length of the tail in an α-helical conformation (1.5 Å per residue), represented by the outer (*red*) and inner (*yellow*) sphere in each panel, respectively. An idealised 30 nm fibre, independent of any connectivity model, is shown with the two nucleosomes rotated by 60° on the fiber axis, and with an internucleosomal rise of 20 Å. Note that the radii of the spheres assume free and unhindered rotation of the tails, which is not always physically possible. The H3 tail, for instance, would have to bend back over the nucleosomal DNA to approach the anterior side of the nucleosome on the "outside" of the fibre, a geometric path that would significantly decrease its reach in that direction

2.2.2 Position of the H2A and H2B Tails

Irrespective of differences in connectivity, all models place the site of DNA entry and exit of the nucleosome, pointing inwards towards the fibre axis (Woodcock and Dimitrov 2001). This places the base of the tails at specific spatial positions within the fibre, and imposes a constraint on the possible sites of interaction of the histone tails, both within and between nucleosomes of the same fibre, as well as to different fibres. Figure 2.2 shows the maximal reach of the N-terminal tails of the core

histones with the tail either fully extended, or with the full length in an α-helical conformation in a hypothetical, idealised fibre. This provides the maximal and minimal reach of the tails, respectively. It is clear that both the N-terminal tails of histones H2A and H2B have limited or no access to an adjacent nucleosome in the idealised fibre structure, but may be involved in fibre-fibre contacts, and should be accessible to *trans*-acting proteins, even in the condensed 30 nm fibre. It is therefore interesting that the sterically accessible H2B K123 is ubiquitinated as a prelude to methylation of the K4 in the histone H3 tail.

2.2.3 Position of the H3 Tail

The histone H3 and H4 N-terminal tails appear to be able to contact distal positions within the same nucleosome as well as neighbouring nucleosomes (see Fig. 2.2). The histone H3 tail is the most extensive, and exits the nucleosome between the two DNA superhelical gyres close to the pseudo-dyad axis (Luger et al. 1997). If the H3 tail continued on its exit trajectory, it would point towards the 30 nm fibre axis, and may approach nucleosomes on the other side of the fibre (see Fig. 2.2).

There exists substantial evidence that the lysine-rich tail of linker histone H1 is associated with the inter-nucleosomal linker DNA in the fibre centre (reviewed in Caterino and Hayes 2011). Since fibre compaction required histone H1 (Thoma et al. 1979; Robinson et al. 2008) as well as the N-terminal tail of histone H4 (Dorigo et al. 2003; Robinson et al. 2008), but not the H3 tail, it appears unlikely that the H3 tail contributed to any significant partial charge neutralisation of the linker DNA in the fibre centre, or acted as a nucleosome-nucleosome stabilisation scaffold, such as the H4 tail. Thus, the possibility of the strong binding of the extended histone H3 tail to the DNA in the fibre centre appears remote. In fact, many studies suggested that the H3 tail was readily accessible in chromatin, including in an H1-containing, condensed fibre. In native H1-containing chromatin, the H3 tail remained the most susceptible to trypsin cleavage (Harborne and Allan 1983). It was also shown that recombinant PCAF, which preferentially acetylated K14 of H3 (Schiltz et al. 1999), could still acetylate the H3 tail in condensed chromatin lacking H1 (Herrera et al. 2000). Furthermore, HP1 was specifically bound to the H3 tail tri-methylated at K9 in condensed heterochromatin (Bannister et al. 2001). In a silenced *MAT***a**-specific gene in *Saccharomyces cerevisiae*, Tup1, which bound at a density of two Tup1 molecules per nucleosome (Ducker and Simpson 2000), was associated with the H3 tail in the repressive chromatin structure (Edmondson et al. 1996). Also, a substituted cysteine residue, close to the tip of the H3 tail, could be cross-linked from one oligonucleosome array to another array (Kan et al. 2007).

All these studies are consistent with a histone H3 tail that is exposed for binding by proteins. Thus, the H3 tail may either continue on its exit trajectory and appear on the side of the central, crossed-linker stack, between the two nucleosome helices in the two-start helix model. Alternatively, it could follow a curved path over the

nucleosomal DNA gyre, protruding into the space between two neighbouring nucleosomes in the 30 nm fiber. In either of these two possibilities, the H3 tail could be bound by sequence specific proteins, or the tail could bind to the originating or to an adjacent nucleosome.

2.2.4 Position of the H4 Tail

The location of the H4 N-terminal tail on the lateral surface of the nucleosome places it in a position where it can easily be extended to contact the lateral surface of the adjacent nucleosome in the chromatin fibre (see Fig. 2.2). Such a contact was, in fact, observed in the crystal structure of *Xenopus* histones reconstituted onto human α-satellite DNA repeats (Luger et al. 1997). Clear contacts were observed to an acidic patch on the nucleosome surface, constituted by H2A E56, E61, E64, D90, E91 and E92 as well as H2B E110. The importance of this observed contact was shown by the absolute requirement for an intact H4 tail by a reconstituted fibre to condense fully in the absence of histone H1 (Dorigo et al. 2003). None of the other core histone tails were required for full compaction (Moore and Ausio 1997; Dorigo et al. 2003). Also, nucleosome arrays reconstituted with the human histone variant H2A-Bbd (Chadwick and Willard 2001), which lacks three glutamic acid residues that forms part of the acidic patch of H2A, did not condense to the same degree as nucleosome arrays reconstituted with H2A (Zhou et al. 2007).

Interestingly, the contact of the H4 tail to the lateral surface of a nucleosome does not appear to require a single docking surface, such as the acidic patch. This was shown by a peptide comprised of residues 1–23 of the Kaposi's sarcoma-associated herpes virus latency-associated nuclear antigen (LANA). When LANA was bound to the acidic patch of a nucleosome (Barbera et al. 2006), this association did not abolish the histone H4-dependent compaction of a nucleosome array (Chodaparambil et al. 2007), suggesting that the H4 tail could still bind to the adjacent nucleosome in the presence of bound LANA. This degeneracy in H4 binding was also demonstrated by the non-saturable nature of association of an H4 peptide with the nucleosome surface, suggesting that many binding sites existed for the H4 tail on the lateral nucleosome surface (Chodaparambil et al. 2007). The binding of the LANA peptide, in contrast, was found to be saturable (Chodaparambil et al. 2007). The H4 tail interaction was, nevertheless, sensitive to chemical modification. It was shown that 30% acetylation of the histone H4 N-terminal tail resulted in the inability of a 61-mer nucleosome array, containing linker histone H1, to fully condense *in vitro* (Robinson et al. 2008). Taken together, these studies provide a very strong argument that the N-terminal tail of histone H4 was bound to an adjacent nucleosome in the chromatin fibre, and that this interaction, which could be disrupted by acetylation of H4 K16, was essential to fully condense the chromatin into a 30 nm fibre structure.

2.3 Histone Tail Associations

2.3.1 N-H2A, H2A-C and N-H2B

Numerous studies have made use of chemical cross-linking to identify DNA and protein sites that can be contacted by the histone tails in the nucleosome and in a condensed fibre by using reagents that are either freely diffusible (Sperling and Sperling 1978; Jackson 1999) or immobilised (Lee and Hayes 1997). The Hayes group have developed a technique where a photo-activatable azidophenacylbromide (ACP) is linked to a uniquely engineered cysteine residue (Lee and Hayes 1997). The conjugated ACP group forms a reactive nitrene upon UV irradiation, cross-linking to spatially proximal DNA or protein molecules, and allowing the mapping of the contact positions of the region containing the substituted cysteine (Lee and Hayes 1997). Using this technique, it was shown that the conjugated A12C of H2A cross-linked to approximately symmetrical positions 4 helical turns removed from the pseudo-dyad in reconstituted and purified nucleosome cores (Lee and Hayes 1997). This is expected from the close proximity of H2A A12 to the DNA in the crystal structure (Davey et al. 2002). In a reconstituted di-nucleosome, cross-linking of residue 12 of the H2A tail was almost exclusively within the same nucleosome (Zheng and Hayes 2003). The more distal portion of the H2A tail, mapped with a G2C substitution, was found to cross-link to two sites approximately 5 bp to either side of the A12C cross-linking position, in agreement with a less constrained motion of the tail further removed from the relatively immobile tail base (Lee and Hayes 1997). This larger freedom of movement of the tail tip was also consistent with the cross-linking of almost 20% of H2A residue 2 to the neighbouring nucleosome in a di-nucleosome template (Zheng and Hayes 2003). Using a zero-length cross-linker, Bradbury and colleagues showed that the H2A C-terminal tail could be cross-linked to the DNA at the pseudo-dyad axis (Usachenko et al. 1994), in agreement with the exit location of this tail from the nucleosome (Luger et al. 1997). Residue 2 of H2B was shown to participate in inter-nucleosomal contacts (Zheng and Hayes 2003).

2.3.2 N-H3

The N-terminal tails of histone H3 and H4 made predominantly intra-nucleosomal contacts in a reconstituted di-nucleosome (Zheng and Hayes 2003). In a 13-mer nucleosome array, it was also found that the H3 tail was exclusively cross-linked intra-nucleosomally at 0 mM Mg^{2+}, but at higher concentrations of Mg^{2+}, where the 13-mer array became more condensed, an increase in inter-nucleosomal cross-links were observed (Zheng et al. 2005). A large proportion of the H3 tail, spanning residues 6–24, could be inter-nucleosomally cross-linked, but not the region of residue 35, close to the base of the tail, which is in agreement with the probable reach of these

regions in the H3 tail (Zheng et al. 2005). Looking at the ability of the H3 tail to make contacts between different nucleosome array molecules, it was found that the entire region spanning residue 6–35 could be efficiently cross-linked within the same reconstituted 12-mer oligonucleosome. Long-range inter-array cross-linking was only detected at higher Mg^{2+} concentrations, ionic conditions suggested to promote self-association of the individual arrays (Kan et al. 2007). This inter-array cross-linking efficiency was increased by the presence of H1, the binding of which may have limited unproductive associations of the H3 tail (Kan et al. 2007). As expected, distal parts of the H3 tail could be cross-linked more efficiently to neighbouring nucleosome arrays compared to regions close to the tail base (Kan et al. 2007). Acetylation of the H3 tail, studied in K->Q substitution mutants, required at least 4 modified residues to display a reduced inter-array cross-linking efficiency, an effect that disappeared at elevated Mg^{2+} concentrations (Kan et al. 2007). The intra-array cross-linking did not appear sensitive to the K->Q substitutions (Kan et al. 2007).

2.3.3 N-H4

The Mirzabekov group showed that H18 of H4 could be cross-linked to the DNA approximately 15 bp from the pseudo-dyad in a nucleosome core particle (Ebralidse et al. 1988). More recently, using reconstituted nucleosome arrays, it was shown that at 0 mM Mg^{2+} the H4 tail cross-linked exclusively within the originating array (Kan et al. 2009). An increase in inter-array cross-links was observed at elevated Mg^{2+} concentrations (Kan et al. 2009). Although an H2A-H4 cross-link, expected from binding of the H4 tail to the H2A-H2B acidic patch, was demonstrated by the simultaneous appearance of fluorescently labelled H2A and tritiated H4 in a higher mobility electrophoretic band, this band was also present in cross-linked mononucleosomes, suggesting that this interaction also occurred intra-nucleosomally (Kan et al. 2009). This cross-link was severely diminished by the presence of the LANA peptide, previously shown to bind in the H2A-H2B acidic pocket (Barbera et al. 2006; Chodaparambil et al. 2007). Interestingly, although tetra-acetylation of H4 reduced fibre self-association, no acetylation dependent difference in inter-fibre cross-linking efficiency was detectable (Kan et al. 2009). In the presence of H1, however, inter-fibre cross-linking was enhanced, and a clear decrease was detected with tetra-acetylated H4 tail (Kan et al. 2009).

2.4 Histone Tail Structure

Many different techniques have been used to study the structure of the histone N-terminal tails. These include the biophysical methods of circular dichroism (CD), nuclear magnetic resonance (NMR) and other forms of spectrometry, and computational methods including secondary structure prediction and molecular dynamics (MD).

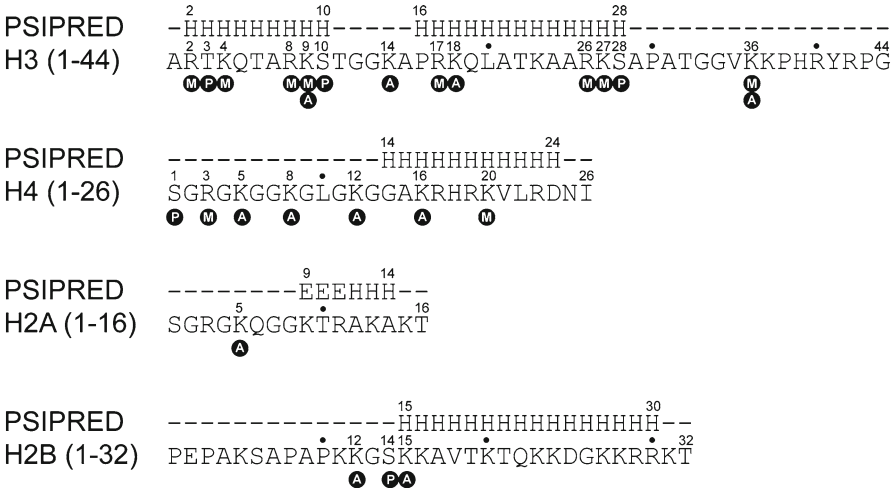

Fig. 2.3 Predicted secondary structures of the core histone tails. The secondary structures predicted by PSIPRED (Jones 1999) are shown above the sequence of each of the four core histone tails with α-helix, β-strand, and random coil regions represented by the symbols "H", "E" and "-", respectively. Sites of epigenetic modification as well as the types of modification are indicated

2.4.1 Secondary Structure Prediction

Secondary structure predictions are often used to obtain insight into the secondary structures of proteins of unknown structure based solely on sequence, and have predictive accuracies in excess of 75% (McGuffin et al. 2000) that are continually being improved by algorithmic advances. The secondary structure predictions for the unmodified, major human core histones using PSIPRED (Jones 1999) are shown in Fig. 2.3.

Two α-helical segments are predicted for H3 spanning 9 residues from R2 to S10, and 13 residues from P16 to S28, respectively. Interestingly, known post-translational modifications (PTMs) appear to be clustered at the predicted α-helix termini, and in both bases serine, which can be phosphorylated (Wei et al. 1998; Goto et al. 1999), are present at the C-terminal end of the predicted α-helices.

In the case of H4 a single 11-residue α-helical segment is predicted spanning from G14 to D24. This segment contains K16, known to be required in a de-acetylated state to allow condensation of the 30 nm fibre *in vitro* (Robinson et al. 2008).

A 3-residue β-strand segment from K9 to R11 followed by a 3-residue α-helical segment spanning A12 to A14 is predicted for the H2A tail, and a single 16 residue α-helical segment is predicted, stretching from K15 to R30, in the case of H2B.

It is therefore clear, based on the propensity of amino acid residues to assume defined secondary structures, that the N-terminal tails of the core histones are likely to be highly structured.

2.4.2 Molecular Dynamics

MD is a molecular mechanics technique that involves the modelling of molecular systems using potential energy functions, and has been widely applied to biomolecular systems over the last 30 years, prominently so in the study of protein folding pathways (Adcock and McCammon 2006).

The application of MD in elucidating the structure of N–terminal histone tails has been limited, and has only been applied to the H3 and H4 tails at the time of writing. Most early work was based on coarse-grained models (Arya and Schlick 2006; Korolev et al. 2006) that were used to study chromatin folding, and did therefore not provide any structural detail on the histone tails. Recently there has been an increase in all-atom MD studies of the tails, and with the development of force field parameters for most of the predominant PTMs (Grauffel et al. 2010), more studies are likely to follow.

LaPenna and co-workers simulated a 25-residue H3 tail peptide in the presence and absence of 10 bp of DNA (LaPenna et al. 2006). The peptide exhibited a wide range of structures with a high α– and 3_{10}-helical content in the presence of DNA. In agreement with the secondary structure prediction (see Fig. 2.3), most of the residues, except for residues 10–15, were found in a helical structure. No β-strand content was observed. The presence of DNA increased the average helical content in the peptide, and resulted in compact, rod-like structures, despite only 4–5 bp of DNA directly interacting with the peptide (LaPenna et al. 2006).

Liu and Duan incorporated PTMs into their MD study of the H3 tail (Liu and Duan 2008), using an 18-residue H3 variant identical to the major H3, except for 2 N-terminal glycine residues. Five PTM states were studied in the H3 peptide: unmodified, K4me2, K9me2, K4me2-K9me2, and K4Ac-K9Ac-K14Ac. The peptides preferred α-helical regions with a similar structure: a shared α-helix between K9 and T11 with the rest in an extended conformation. The singly di-methylated peptides did not differ significantly from the unmodified peptide. The doubly dimethylated peptide, however, showed a decrease in α-helical and an increase in β-strand content, although the biological relevance of a simultaneous K4 and K9 methylation is questionable. The acetylated peptide showed a decrease in helical content compared to the unmodified peptide, and exhibited a β-hairpin as the most populated structure (Liu and Duan 2008). It is thus evident that "cross-talk" between different modification groups may have a structural basis, where combinations of modifications may stabilize specific secondary structural distributions in the tail that could influence binding of the tails in chromatin.

Lins and Röthlisberger conducted MD studies on tetra- and un-acetylated 23-residue N-terminal H4 peptides (Lins and Röthlisberger 2006). The starting conformation for the two peptides was a canonical α-helix, which was found to be more stable in the tetra–acetylated peptide than in the un-acetylated peptide. A small β-hairpin was formed that spanned residues 4–12 in the tetra-acetylated peptide, which remained stable for approximately 2 ns of a 20 ns simulation (Lins and Röthlisberger 2006). Taken together with results from the previous studies, the

histone tails seem able to stably accommodate secondary structures other than only α-helices. This opens the possibility that modifications to residues may be a way of changing the transition of the tails to different secondary structures on the fly, impacting on tail binding and, consequently, chromatin structure, and could thus provide a mechanism for genetic control.

In the most recent MD study, Yang and Arya investigated the effect of K16 acetylation in a 25-residue H4 tail peptide (Yang and Arya 2011). An α-helical region was formed and stabilized between residue 15 and 20 in the unmodified peptide. An α-helix was formed in the same region in the K16Ac peptide, but, in contrast to another study (Lins and Röthlisberger 2006), the helix exhibited a significantly reduced stability (Yang and Arya 2011). It is, however, important to note that the authors of the MD studies used a wide range of different simulation protocols and techniques, which makes the comparison of results between studies difficult.

Nevertheless, MD studies suggested that both H3 and H4 tail peptides preferred helix-rich structures. PTMs changed the stability of these structures, and β-strands were also observed in some cases. These studies therefore underscore a possibly critical role in PTMs tipping the balance between different secondary structures in the histone tails, which may have a major impact on the function of these tails.

2.4.3 Biophysical Methods

Parello and co-workers compared CD spectra obtained from a native and two selectively proteolyzed nucleosome core particles (NCP) to investigate the secondary structure of the N-terminal tails (Banères et al. 1997). Clostripain was used to produce a "half-proteolyzed" NCP that lacked the H3 and H4 tails, and a "fully proteolyzed" NCP, that lacked all four core histone tails. The authors established that approximately 60% of the residues in the H3 and H4 tails were in an α-helical conformation, and contributed about 35% to the α-helical content in the whole NCP. It was confirmed that these contributions corresponded to the tails in the bound state in the nucleosome. The individual contributions of the H3 and H4 tails to α-helical content could, however, not be resolved. The H2A and H2B tails were found to be in a random coil conformation. A subsequent NMR study also showed that 31 residues of the H2B tail were unstructured (Nunes et al. 2009).

Ausio and co-workers investigated the contribution of the histone tails to the secondary structure of the octamer, and the effect that acetylation of the tails had on this contribution (Wang et al. 2000). The contribution of the tails to the overall α-helical content of the octamer was calculated at 17% by comparing the α-helical content of trypsin digested octamer with an undigested octamer. This value was about half of that reported by Parello and colleagues (Banères et al. 1997), and was attributed to the use of different experimental conditions. Consequently, it was shown that the overall α-helical content of the nucleosome increased by about 3% as a result of acetylation. This translated to an increase of about 17% in the α-helicity of the tails. An H4 tail peptide corresponding to residue 1–23 was isolated as

mono-, di-, tri- and tetra-acetylated isomers, and analysed by CD in an aqueous solution and in trifluoroethanol (TFE), a known stabilizer of α-helices. The unmodified peptide showed an α-helical content of 17% in TFE, which increased to about 24% in the tetra-acetylated peptide in the same solvent. In the aqueous solution, the isolated peptides exhibited CD spectra consistent with a random coil conformation, suggesting that the chemical environment of the histone tails played a major role in their structural conformations.

In a combined NMR and CD study Lee and co-workers also showed that a 27-residue synthetic H4 peptide had no defined structure in aqueous solution at physiological pH (Bang et al. 2001). However, a pH dependent structural transition was observed at an acidic pH for the native peptide. None of the peptides displayed any regular secondary structures. The acetylated form of this peptide seemed insensitive to pH change, and exhibited two regions of turn-like structures at L10-G13 and R19-L22.

2.5 Histone Tails and Human Health

A link between chromatin and human disease is long established. In recent times thousands of studies have been published reporting the role of epigenetics in human disease. This role is varied and fundamental. Epigenetics was shown to be involved in development, trans-generational inheritance, memory formation, psychiatric disorders, autism spectrum disorders, carcinogenesis, cardiovascular diseases and a slew of heritable diseases including Fragile X syndrome, Friedreich's ataxia, Machado-Joseph disease, spinocerebellar ataxia, Huntington's disease and myotonic dystrophy, to provide but a significantly truncated representative list. Epigenetics have also been implicated in longevity in model eukaryotic organisms (Dang et al. 2009). Many excellent reviews have recently appeared on epigenetics and human health (Watanabe and Maekawa 2010; Luco et al. 2011; Kurdistani 2011). Because of the extensive role of epigenetics in human disease, modulators of epigenetic modifications suitable for therapy have become pharmacologically highly prized (Kundu 2007). A multitude of modifiers, including deactylase and demethylate inhibitors, are currently in various phases of clinical trials, and many show extremely promising results.

Many of the epigenetic therapeutic agents direct a change in gene expression level of numerous genes, where misexpression is associated with a diseased state. The precise mechanism whereby the epigenetic modification alters gene expression level is often not fully understood. Some modifiers are now known to induce structural transitions in the core histone tails. For instance, the binding of Ni^{2+} to the sequence 15-AKRHRK-20 in the tail of H4 showed a drastic structural shift in the conformation of the peptide (Zoroddu et al. 2000). The binding of Ni^{2+} to a 22-residue H4 tail peptide had the same effect as acetylation on the α-helical content of the peptide (Zoroddu et al. 2009). This is an interesting observation since Ni is a known carcinogen which seems to act on the epigenetic level. This suggests that

the epigenetic link between some human diseases and chromatin may not simply be the chemical modifications of the core histone tails that subsequently act as binding surfaces for transcription-related enzymes, but may also occur due to changes in the stable secondary structures of the histone tails which may impact not only on transcription, but also other genetic processes of the DNA molecule.

2.6 Conclusions

There is significant evidence that the core histone tails are partially structured (Banères et al. 1997; Wang et al. 2000), and that they are involved in intra- and internucleosomal as well as in inter-fibre contacts (Zheng et al. 2005; Kan et al. 2007, 2009). It seems likely that the H4 tail binds to the lateral surface of an adjacent nucleosome in chromatin (Luger et al. 1997), and may act as a molecular tether, stabilising the architecture of the 30 nm fibre (Dorigo et al. 2003; Robinson et al. 2008). It is further known that acetylation of H4 K16 abolished formation of the 30 nm fibre (Robinson et al. 2008). Although this may simply involve a reduced electrostatic attraction between the acidic surface and the acetylated lysine residue, it is also possible that acetylation may disrupt secondary structures required for docking to the acidic patch or to sites in its vicinity. Alternatively, acetylation may stabilise an extended α-helix, diminishing the reach of the H4 tail, and limiting contact to the adjacent nucleosome.

Although no H3 mediated inter-nucleosome contacts were seen in X-ray crystallographic studies, this tail was, nevertheless, shown to bind intra-nucleosomally as well as between fibres (Zheng et al. 2005; Kan et al. 2007). The predicted presence of two α-helices, demarcated by clusters of sites targeted for epigenetic modification, appears intriguing. Although, clearly, the recognition and binding of specific protein domains such as chromo and bromo domains to methylated and acetylated lysine residues are well established, and recruit proteins that serve crucial biochemical functions, the cross-linking data suggests that the H3 as well as the H2A and H2B tails are also involved in binding to DNA and/or protein surfaces in chromatin (Lee and Hayes 1997; Zheng and Hayes 2003; Kan et al. 2007). The binding of chromatin-associated proteins and enzymes to the histone tails may therefore only reflect a part of the functionality of the tails, which may also make a direct structural contribution to chromatin organization. One may therefore speculate that specific PTMs, stabilizing a specific distribution of secondary structures, are required for binding of the tail in chromatin. Removal of these PTMs may destabilise the structure, disrupt binding, and allow subsequent association of other regulatory proteins with the released tail. Conversely, specific PTMs may favour defined structures that allow an exact binding in chromatin, which may then provide a combined molecular surface that is recognised and bound by other regulatory factors. It is thus evident from the studies cited above that our understanding of the biochemical role of the core histone tails is incomplete, and that the tails may be multi-functional molecular entities that impact on chromatin structure and genetic function in a way that is only

partially appreciated. This opens the exciting possibility of a different angle on the role of epigenetics in human disease, and the development of therapies that target histone tail structures and patterns of association as opposed to only the enzymes that are recruited by a fraction of the epigenetic marks.

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Chapter 3
Megabase Replication Domains Along the Human Genome: Relation to Chromatin Structure and Genome Organisation

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Abstract In higher eukaryotes, the absence of specific sequence motifs, marking the origins of replication has been a serious hindrance to the understanding of (i) the mechanisms that regulate the spatio-temporal replication program, and (ii) the links between origins activation, chromatin structure and transcription. In this chapter, we review the partitioning of the human genome into megabased-size replication domains delineated as N-shaped motifs in the strand compositional asymmetry profiles. They collectively span 28.3% of the genome and are bordered by more than 1,000 putative replication origins. We recapitulate the comparison of this partition of the human genome with high-resolution experimental data that confirms that replication domain borders are likely to be preferential replication initiation zones in the germline. In addition, we highlight the specific distribution of experimental and numerical chromatin marks along replication domains. Domain borders correspond to particular open chromatin regions, possibly encoded in the DNA sequence, and around which replication and transcription are highly coordinated. These regions also present a high evolutionary breakpoint density, suggesting that susceptibility to breakage might be linked to local open chromatin fiber state. Altogether, this chapter presents a compartmentalization of the human genome into replication domains that are landmarks of the human genome organization and are likely to play a key role in genome dynamics during evolution and in pathological situations.

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3.1 Introduction

DNA replication is an essential genomic function responsible for the accurate transmission of genetic information through successive cell generations. According to the so-called "replicon" paradigm derived from prokaryotes (Jacob et al. 1963), this process starts with the binding of some "initiator" protein to a specific "replicator" DNA sequence called *origin of replication*. The recruitment of additional factors initiate the bi-directional progression of two divergent replication forks along the chromosome. In eukaryotic cells, this event is initiated at a number of replication origins and propagates until two converging forks collide at a *terminus of replication* (Bell and Dutta 2002). In general, metazoan replication origins are rather poorly defined and initiation may occur at multiple sites distributed over a thousand of base pairs (Gilbert 2001). Moreover, random initiation of replication was repeatedly observed in *Drosophila* and *Xenopus* early embryo cells, suggesting that any DNA sequence can function as a replicator (Hyrien and Méchali 1993; Coverley and Laskey 1994; Sasaki et al. 1999). Thus, although it is clear that some sites consistently act as replication origins in most eukaryotic cells, the mechanisms that select these sites and the sequences that determine their location remain elusive in many cell types (Bogan et al. 2000; Gilbert 2004). The need to fulfill specific requirements that result from cell diversification may have led multicellular eukaryotes to develop various epigenetic controls over the replication origin selection rather than to conserve specific replication sequence (Méchali 2001; Demeret et al. 2001; McNairn and Gilbert 2003).

In that context, we report on what we learned about the human replication program starting from an *in silico* analysis of strand composition asymmetry (skew) profiles (Brodie of Brodie et al. 2005; Touchon et al. 2005). We describe a wavelet-based method to systematically detect replication domains labeled N-domains as their skew profile displays a N-like shape attributed to mutational asymmetries intrinsic to the replication process (Arneodo et al. 2007, 2011; Audit et al. 2007; Huvet et al. 2007; Baker et al. 2010; Chen et al. 2011). We present a comparative analysis of the detected N-domains with experimental replication data, in particular with preferential replication initiation zones identified by a multi-scale peak detection analysis of high-resolution replication timing profile (Chen et al. 2010; Hansen et al. 2010). This study corroborates N-border as putative replication origins of the germline. Finally, we examine the distribution of genes and of numerical and experimental chromatin marks within N-domains. Altogether these results make a compelling case that N-domain borders are "islands" of open chromatin defining units of coordinated replication and gene transcription.

3.2 Uncovering Replication Skew N-domain Along the Human Genome

The existence of replication associated strand asymmetries has been mainly established in bacterial genomes (Lobry 1995, 1996; Mrázek and Karlin 1998; Frank and Lobry 1999; Rocha et al. 1999; Tillier and Collins 2000). $S_{GC} = (G-C)/(G+C)$

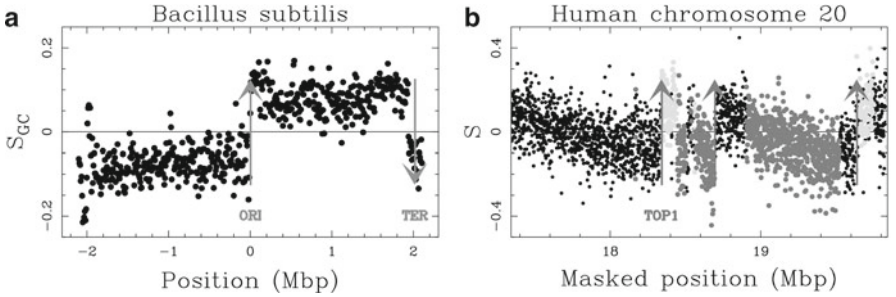

Fig. 3.1 (**a**) Skew profile S_{GC} along the genome of *Bacillus subtillis* calculated in 8192 bp windows. The *upward* (resp. downward) *arrow* marks the unique origin of replication (ORI) (resp. terminus (TER)) where a transition from negative to positive (resp. positive to negative) skew values is observed (Lobry 1996). (**b**) Skew profiles $S = S_{TA} + S_{GC}$ along a fragment of chromosome 20 including the TOP1 origin calculated in 1 kbp windows of the repeat-masked sequence (Touchon et al. 2005; Arneodo et al. 2007). *Upward arrows* mark upward jumps in the skew profile that have been identified as putative replication origins (Brodie of Brodie et al. 2005; Touchon et al. 2005). *Black*, intergenic regions; *light gray*, sense (+) genes; *dark grey*, antisense (−) genes

and $S_{\mathrm{TA}} = (T - A)/(T + A)$ skews abruptly switch sign (over few kbp) from negative to positive values at the replication origin and in the opposite direction from positive to negative values at the replication terminus. This step-like profile is characteristic of the replicon model (Jacob et al. 1963) (Fig. 3.1a). In eukaryotes, the existence of compositional biases is unclear. Most attempts to detect the replication origins from strand compositional asymmetry have been inconclusive (Mrázek and Karlin 1998; Bulmer 1991; Francino and Ochman 2000). Even though, strand asymmetries associated with replication have been observed in the subtelomeric regions of *Saccharomyces cerevisiae* chromosomes (Gierlik et al. 2000).

As shown in Fig. 3.1b for TOP1 replication origin (Brodie of Brodie et al. 2005; Touchon et al. 2005; Arneodo et al. 2007, 2011), most of the experimentally known replication origins in the human genome correspond to rather sharp (over several kbp) transitions from negative to positive skew values (S_{TA}, S_{GC} as well as $S = S_{TA} + S_{GC}$) that clearly emerge from the noisy background. Moreover, sharp upward jumps of amplitude $\Delta S > 15\%$, similar to the ones observed for the known replication origins, also exist at many other locations along the human chromosomes (Figs. 3.1b and 3.2a). But the most striking feature is the fact that in between two neighboring major upward jumps, not only does the noisy S profile not present any comparable downward sharp transition as observed for the bacterial replicon (Fig. 3.1a), rather it displays a remarkable decreasing linear behavior. At chromosome scale, we thus get jagged *S* profiles that have the aspect of a succession of Ns (Brodie of Brodie et al. 2005; Touchon et al. 2005; Arneodo et al. 2007). In genic regions, we observe the superimposition of this replication N-shaped profile and of the step-like profiles resulting from transcription associated strand asymmetry (Touchon et al. 2003, 2004; Nicolay et al. 2007; Baker et al. 2010), that appear as upward ((+) genes) and downward ((−) genes) blocks standing out from the replication pattern (see Fig. 3.1b, rhs of TOP1). The observation that some of the segments between two successive skew upward jumps are entirely intergenic (Fig. 3.1b, lhs of

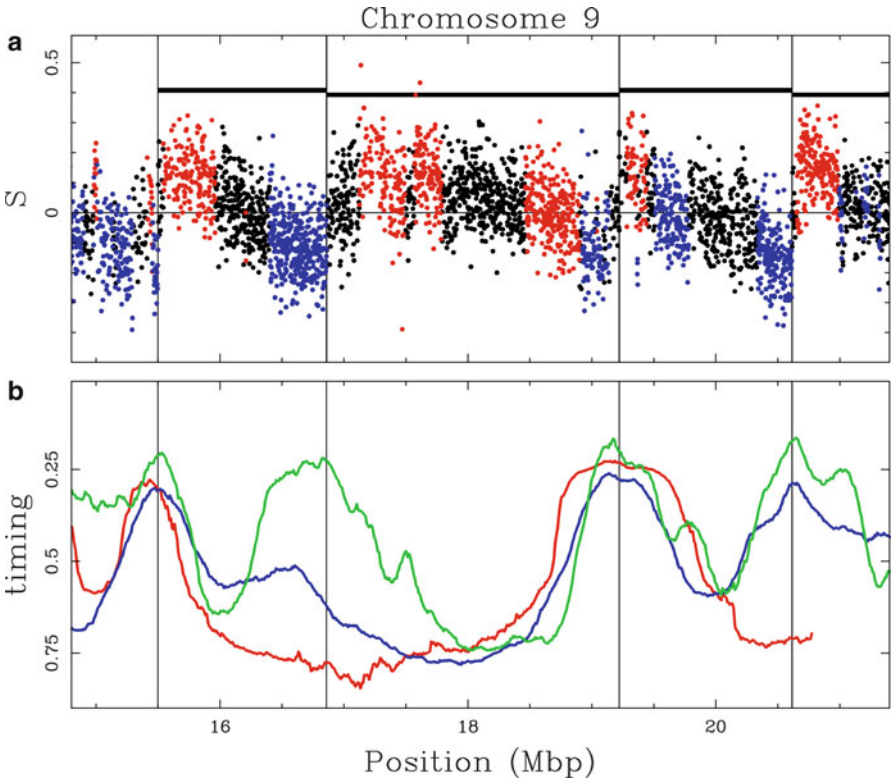

Fig. 3.2 Compositional skew and replication timing profiles along a fragment of Human chromosome 9. (**a**) Skew profiles $S = S_{TA} + S_{GC}$ calculated in 1 kbp windows of the masked-sequence (Touchon et al. 2005; Arneodo et al. 2007, 2011). *Horizontal black lines* mark the replication N-domains delineated using a multi-scale methodology (Sect. 3.6.1) (Audit et al. 2007; Huvet et al. 2007; Baker et al. 2010). *Vertical lines* mark the corresponding putative replication initiation zones. *Black*, intergenic regions; *red*, sense (+) genes; *blue*, antisense (−) genes. (**b**) Mean replication timing determined in BG02 ESC (*green*), K562 (*red*) and GM06990 (*blue*) cell lines (Hansen et al. 2010)

TOP1), clearly illustrates that the N-shape profiles result solely from replication and not from some particular distribution of transcription units (Brodie of Brodie et al. 2005; Touchon et al. 2005; Arneodo et al. 2007, 2011; Huvet et al. 2007). Importantly, it is observed that strand asymmetry profiles are conserved between a number of human regions and their homologous loci in the mouse and dog genomes (Touchon et al. 2005). This conservation between regions that have strongly diverged during evolution further supports the association between N-shaped skew profiles and replication in mammalian germline cells (Brodie of Brodie et al. 2005; Touchon et al. 2005; Arneodo et al. 2007, 2011). On shorter evolutionary time scale, recent computation of nucleotide substitution rates in the human lineage since divergence with chimpanzee shows that the differences between complementary rates (mutational asymmetry between the complementary strands) change sign when crossing

replication initiation zones identified as abrupt upward jumps in the skew profile (Chen et al. 2011). This analysis demonstrates that the mutational patterns observed in these regions fully explain the jumps in the *S* skew profile and provides strong evidence in favor of a model based on replication errors and strand-biased repair to explain the origins of N-domains.

We developed a multi-scale procedure to detect replication N-domains along the skew *S* profiles (Sect. 3.6.1). As illustrated in Fig. 3.2a for a fragment of human chromosome 9 that contains adjacent replication domains, this wavelet-based methodology provides a very efficient way of delineating the N-shaped components induced by replication along the skew *S* profile. Applying this procedure to the 22 human autosomes from human May 2004 (hg17) assembly, we delineated 678 replication domains of mean length $L = 1.2 \pm 0.6$ Mbp, containing 18% of the genes (Audit et al. 2007; Huvet et al. 2007; Baker et al. 2010). There are 1,060 different N-domains borders since in 296 cases a border in shared by two consecutive domains. Here, we used the LiftOver coordinate conversion tool from the UCSC website to map N-domains to Human March 2006 (hg18) coordinates and we kept only the N-domains that had exactly the same size before and after conversion. This resulted in the assignment on hg18 of 663 unambiguous N-domains delimited by 1,040 borders and spanning 28.3% of the sequenced genome length. To our knowledge, these loci correspond to the largest set of human replication initiation zones available to date (Baker et al. 2010).

3.3 DNA Replication Data Corroborate Human Replication Skew N-domain Predictions

3.3.1 N-domain Borders Correspond to Experimental Replication Origins Mapped on ENCODE Pilot Regions

Previous analyses of nucleotide strand compositional asymmetries have shown that, out of the 9 experimentally identified replication origins, 7 (78%) presented an upward jump in the asymmetry profile analog to those bordering N-domains (Brodie of Brodie et al. 2005; Touchon et al. 2005). Recently, the localization of replication origins has been experimentally investigated along 1% of the human genome (ENCODE pilot regions) by hybridization to Affymetrix ENCODE tiling arrays of purified small nascent DNA strands and of restriction fragments containing small replication bubble (The ENCODE Project Consortium 2007; Cadoret et al. 2008). Out the 7 N-domain borders that reside within an ENCODE region, 4 match an experimental replication origin ($P = 4\,10^{-3}$): 3 to the bubble trapping dataset (Bubble-HL, $P = 0.017$) and 1 to a nascent strand purification dataset (NS-HL-2, $P = 0.09$) (Table 3.1). Actually, as previously noted (Cadoret et al. 2008), a second N-domain border is located within 1 kbp of a NS-HL-2 origin (Table 3.1). Hence, there is direct experimental evidence that 5/7 (71%) N-domain borders

Table 3.1 Correspondence between N-domain borders and experimental replication origins datasets along ENCODE pilot regions (The ENCODE Project Consortium 2007; Cadoret et al. 2008)

Method	Number	Coverage	Match with N-domain borders (P-value)
Bubble-HL	234	8.6%	3 (**0.017**)
NS-GM	758	1.0%	0
NS-HL-1	434	0.6%	0
NS-HL-2	282	1.4%	1 (0.093)
All		11%	4 (**0.004**)
NS+1kbp-HL-2		3.2%	2 (**0.019**)
+Bublle-HL		11.2%	5 (**0.0003**)

Main characteristics of replication origin prediction along ENCODE pilot regions based on purified restriction fragments containing replication bubble (Bubble) (Mesner et al. 2006) or purified small nascent strands (NS) (Gerbi and Bielinsky 1997). First column indicates the experimental method (Bubble or NS) and the cell type (HL: HeLa cells and GM: GM06990 cell lines). They are two independent NS-HL datasets labelled 1 and 2. "All" corresponds to the four initial datasets considered together. NS+1kbp-HL-2 corresponds to the NS-HL-2 dataset when extending replication origins by 1 kbp on both sides. +Bublle-HL corresponds to merging the NS+1kbp-HL-2 and Bubble-HL datasets. We provide the number of replication origins, their total coverage of ENCODE pilot regions, the number of N-domain borders out of 7 within ENCODE regions that match with one of the experimental replication origins and the corresponding P-value using a binomial test (Audit et al. 2009)

correspond to active replication origins at a few kbp resolution (Audit et al. 2009). These results are all the more significant considering the rather low overlap between the experimental datasets. For example, only 69 (25%) of the NS-HL-2 origins overlap with a Bubble-HL origins, only 4 (1.4%) with the second nascent strand dataset in HeLa cells (NS-HL-1) and only 12 (4.3%) even when extending NS-HL-1 and NS-HL-2 origins by 1 kbp on both sides.

3.3.2 N-domain Borders Colocate with Peaks in Replication TimingProfiles

The recent advance in the experimental characterization of replication programme provides genome-wide timing data in several human cell types (Woodfine et al. 2005; Desprat et al. 2009; Chen et al. 2010; Hansen et al. 2010; Ryba et al. 2010; Yaffe et al. 2010). We examined the average timing profiles computed from Repli-Seq data (Chen et al. 2010; Hansen et al. 2010) for 5 cell lines including one embryonic stem cell line (BG02), a fibroblast (BJ), a lymphoblastoid (GM06990), an erythroid (K562) and an HeLa cell line. They all present numerous peaks pointing towards early replication time (Figs. 3.2b and 3.6a). The regions at the tip of the peaks are on average replicated earlier than their surrounding regions and thus harbor replication initiation zones highly active in the corresponding cell type. In the 6 Mbp regions analyzed in Fig. 3.2, we observed a strong correspondence between the germline putative replication origins at N-domain borders and the initiation zones

Table 3.2 Correspondence between N-domain borders and peaks delineated along genome-wide timing profiles (Chen et al. 2010; Hansen et al. 2010). For each cell line, we indicate the number of timing peaks, the cumulative genome coverage of the regions ± 100 kbp around the peaks, the number of N-domain borders located within these regions and the corresponding proportion of so-confirmed N-domain borders. "All" corresponds to the five timing peak datasets considered together. In the six cases the number of N-domain borders with a distance to a timing peak smaller than 100 kbp is highly significant (P-value $< 10^{-15}$ using a binomial test)

Cell line	Number of timing peaks (Genome cov.)	Number of co-localization with N-domain borders (N-domain border prop.)
BG02	1,690 (12.5%)	333 (32%)
BJ	981 (7.3%)	185 (18%)
GM06990	706 (5.2%)	167 (16%)
Hela	1,556 (11.5%)	292 (28%)
K562	795 (5.8%)	176 (17%)
All	5,728 (28.0%)	590 (57%)

pointed to by the timing peaks. We also noticed a strong conservation of timing peak location between cell lines in this region. In order to perform a systematic comparison between these loci containing strong replication origins and N-domain borders, we developed a multi-scale methodology for the detection of peaks along timing profiles (Sect. 3.6.2). For the 5 cell lines considered, we delineated between 706 (for GM06990) and 1,690 (for BG02) timing peaks (Table 3.2). When comparing the observed distribution of the distances of N-domain borders to the closest timing peak (considering the 5 cell lines) to the expected distribution for uniformly distributed borders, we observed a significant excess of short distances (175 kbp) (Fig. 3.3a). This result provides a first quantitative evidence for the co-localisation of N-domain borders with the active replication initiation zones at timing peaks in different cell lines.

The spatial resolution of the timing profiles being ~100 kbp, we carried on the analysis considering that N-domain borders and timing peaks coincide if they are within 100 kbp from each other. In BG02, the regions ±100 kbp of the timing peaks cover 12.5% of the genome but contain 32% of the N-domain borders. Consistently, we observed a significant over-representation of N-domain borders in the regions ± 100 kbp of timing peaks for all 5 cell lines (Table 3.2). Altogether, 57% of N-domain borders are associated (at 100 kbp resolution) with an active replication initiation regions in at least one of the considered cell lines. Moreover, N-domain borders and BG02 timing peaks present a very similar degree of association with the timing peaks of the 4 other cell lines: 55% (575/1,040) of N-domain borders and 54% (908/1,690) of BG02 timing peaks are not associated within 100 kbp with a peak in one of the other 4 cell lines and only 2.9% (30/1,040) and 3.7% (62/1,690) respectively are also found in the 4 other cell lines. This observation is a strong indication that N-domain borders mark replication initiation zones active in the germline.

Finally, we observed that the probability of BG02 timing peaks to correspond to a N-domain border increases with their conservation level in the 4 other cell lines,

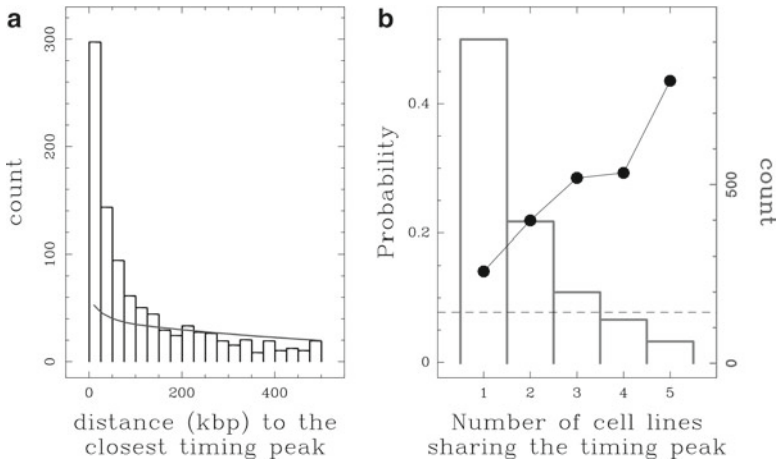

Fig. 3.3 (**a**) N-domain border distances to the closest replication timing peak from one of the 5 cell lines considered; (*black*) the histogram corresponds to observe counts in 25 kbp bins; (*grey*) the *solid line* marks the expected counts for uniformly distributed N-domain borders. (**b**) BG02 timing peaks are classified according to the number of cell lines where a corresponding timing peak is present within 100 kbp (from 1 for BG02 specific peaks to 5 when a peak is observed within 100 kbp in the 4 other cell lines); (*black bullet*) for each BG02 peak category, we report the proportion of peaks corresponding to a N-domain within 100 kbp; the *dashed line* marks the expected proportion (7.7%) for uniformly distributed N-domain borders; (*grey*) the histogram corresponds to the number of BG02 peaks in each category

from 14% for BG02 specific peaks to 44% for peaks also observed in the 4 other cell lines (Fig. 3.3b). Similar results are generally obtained when considering the conservation of timing peaks (Chen et al. 2011). This illustrates that replication initiation zones have very diverse conservation status relative to cell differentiation, from cell specific initiation zones to apparently generally active zones (Hiratani et al. 2008, 2010).

3.4 Open Chromatin Encoded in the DNA Sequence is the Signature of Germline Initiation Zones

Our knowledge of the mechanisms that control the spatio-temporal program of replication remains sparse and understanding how origins are distributed along the genome and how their activation is controlled and coordinated constitutes one of the main challenges of molecular biology (Schwaiger and Schubeler 2006). For years, the small number (~30) of well established origins in the human genome and more generally in mammalian genomes, has been an obstacle to fully appreciate the genome-wide organization of replication in relation to gene expression and local chromatin structure – much remains to be understood about the impact of the DNA sequence on origin activity in human cells in parallel to epigenetic controls (Bogan et al. 2000; Méchali 2001; Gerbi and Bielinsky 2002; McNairn and

Gilbert 2003; Lemaitre et al. 2005; Courbet et al. 2008; Hamlin et al. 2008). In that context, the 663 N-domains provide an unprecedented opportunity to analyze the human replication program in relation to chromatin state and gene organization (Huvet et al. 2007; Audit et al. 2009; Arneodo et al. 2011). Indeed as it has been argued in the previous section, the comparison of recent high-resolution replication data with N-domains provided clear experimental evidence for their relationship to the replication program (Audit et al. 2007; Huvet et al. 2007; Chen et al. 2011). N-domains borders are associated to regions that replicate earlier in the S phase than their surrounding regions and are thus likely associated with early replicating origins, while N-domain central regions are late replicating (Fig. 3.2). Hence, most N-domains correspond to units of replication where timing decreases when going from borders to center (Audit et al. 2007; Huvet et al. 2007; Baker et al. 2012).

3.4.1 Active Replication Initiation Regions at N-domain Borders are Hypersensitive to DNase I Digestion

High-throughput sequencing and whole-genome tiled strategies have been developed to identify DNase I hypersensitive sites (HS) as markers of open chromatin across the genome (Sabo et al. 2006; Boyle et al. 2008). When mapping DNase I HS determined in GM06990 inside the 663 replication N-domains, we observed that the mean site coverage is maximum at the N-domain extremities and decreases significantly from the extremities to the center that is rather insensitive to DNase I cleavage (~1/3 of the genome average; Fig. 3.4a, b). This decrease extends over ~150 kbp whatever the size of replication N-domains suggesting that N-domain extremities are at the center of an open chromatin region with a ~300 kbp mean characteristic size (Audit et al. 2009). We observed that the over representation of GM06990 DNase I HS is much stronger at the 167 N-domain borders that co-localize (±100 kbp) with a GM06990 timing peak (3.5 the genome average; Fig. 3.4a) compared to the 873 other N-domain borders (1.5 the genome average; Fig. 3.4b). These results illustrate the dynamic coupling between the activity of a replication initiation zones and the chromatin status of the surrounding region (Baker et al. 2012).

3.4.2 DNA Sequence Codes for the Accumulation of Nucleosome Free Regions Around N-domain Borders

Previous analysis revealed that promoter regions for protein-coding genes are extremely hypersensitive to DNase I digestion (Boyle et al. 2008). These regions were shown to be nucleosome depleted (Boyle et al. 2008; Ozsolak et al. 2007; Heintzman et al. 2007; Barski et al. 2007; Schones et al. 2008), very much like the NFRs observed at yeast promoters (Lee et al. 2007; Yuan et al. 2005). Recent numerical studies revealed that, to a large extent, these NFRs are coded in the DNA

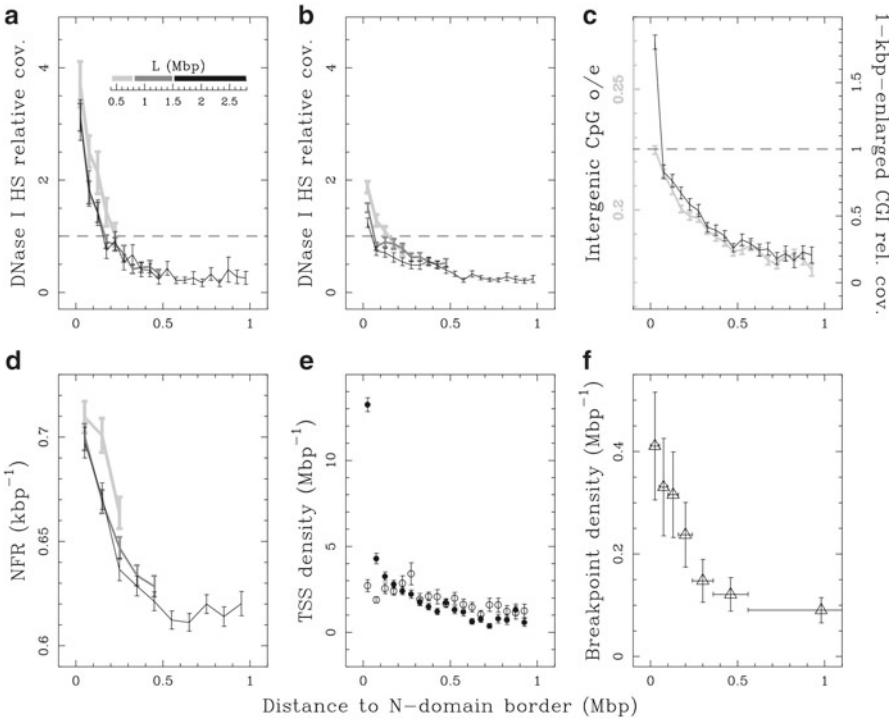

Fig. 3.4 Genome organization and chromatin marks distribution within N-domains. (**a, b**) DNase I hypersensitive sites (HS) coverage relative to the genomic average. DNase I sensitivity data were obtained in GM06990 cell line; N-domain borders were classified accordingly: N-domain borders corresponding to a GM06990 timing peak in (**a**) and other N-domain borders in (**b**) (Table 3.2); the three shades of *grey* corresponds to three N-domain size categories: $L<0.8$ Mbp (*light grey*), $0.8<L<1.5$ Mbp (*dark grey*) and $L>1.5$ Mbp (*black*); for each dataset, the average profile determined in 50 kbp windows is plotted as a function of the distance to the closest N-domain border; the *horizontal dashed line* represent the genomic average of DNase I HS coverage. (**c**) 1-kbp-enlarged CGI coverage normalized by the genomic average (*black*) and CpG ratio computed for intergenic regions (*grey*) determined in 50 kbp windows; the average profiles are plotted as a function of the distance to the closest N-domain border; the *horizontal dashed line* represent the genomic average of 1-kbp-enlarged CGI coverage. (**d**) In silico NFR density determined in 50 kbp windows with GC content $<41\%$; the average profiles are plotted as a function of the distance to the closest N-domain border for the same N-domain size categories as in (**a, b**). (**e**) Average transcription start site (TSS) density determined in 50 kbp windows as a function of the distance to the closest N-domain border for CpG rich (*dots*) and CpG poor (*circles*) TSS. (**f**) Intergenic breakpoint density as a function of the distance to the closest N-domain border; horizontal bars represent the distance range used for each data point such that they all correspond to a ~equal number of intergenic breakpoints. In (**a–f**) vertical bars represent standard errors of the mean

sequence via high energy barriers that impair nucleosome formation (Vaillant et al. 2007; Miele et al. 2008; Mavrich et al. 2008; Chevereau et al. 2009; Vaillant et al. 2010). Furthermore, these excluding genomic energy barriers were shown to play a fundamental role in the collective nucleosomal organization observed over

rather large distances along the chromatin fiber (Vaillant et al. 2007, 2010). Here we used the same physical modeling of nucleosome formation energy based on sequence-dependent bending properties as previously introduced for modeling nucleosome occupancy profiles in the yeast genome (Vaillant et al. 2007; Miele et al. 2008). Since the GC content of *S. cerevisiae* is rather homogeneous around 39% as compared to the heterogeneous isochore structure of the human genome (Bernardi 2001), we restricted our modeling of nucleosome positioning to the light isochores L1 and L2 (GC <41%). Combining the nucleosome occupancy probability profile and the original energy profile, we identified nucleosome NFRs as the genomic energy barriers that are high enough to induce a nucleosome depleted region in the nucleosome occupancy profile. We checked that the average of an experimental genome-wide nucleosome occupancy profile (Schones et al. 2008) presented a clear nucleosomal depletion at the *in silico* predicted NFR positions (Audit et al. 2009). This indicates that the regions depleted in nucleosome in vivo are likely to be encoded, at least to some extent, in the DNA sequence. The distribution of NFRs along the 663 N-domains shows a mean density profile that is maximum at N-domain extremities (~0.7 NFR/kbp) and that decreases from extremities to center where some NFR depletion is observed (~0.62 NFR/kbp) (Fig. 3.4d). This decay over a characteristic length scale ~150 kbp is strikingly similar to that displayed by DNase I HS coverage (Fig. 3.4a, b).

Altogether, these results show that the NFR density profile displays the same characteristic increase around N-domain borders, as the experimental DNase I HS coverage profile (Audit et al. 2009). If this correlation was expected, the fact that we recovered it using a sequence-based modeling of nucleosome occupancy suggests that putative replication origins that border the N-domains are situated within regions of accessible open chromatin state that are likely to be encoded in the DNA sequence via excluding energy barriers that inhibit nucleosome formation and participate to the collective ordering of the nucleosome array (Vaillant et al. 2007, 2010; Arneodo et al. 2011).

3.4.3 DNA Hypomethylation Is Associated with N-domain Borders

Cytosine DNA methylation is a mediator of gene silencing in repressed heterochromatic regions, while in potentially active open chromatin regions, DNA is essentially unmethylated (Bird and Wolffe 1999). DNA methylation is continuously distributed in mammalian genomes with the notable exceptions of CpG islands (CGIs), short unmethylated regions rich in CpGs, and of certain promoters and transcription start sites (TSS) (Suzuki and Bird 2008). Since there was no genome wide map of DNA methylation available, we investigated the distribution of DNA methylation using instead indirect estimators calculated directly from the genomic sequence. Methyl-cytosines being hypermutable, prone to deamination to thymines, we considered the CpG observed/expected (CpG o/e) ratio as an estimator of DNA

methylation (Bird 2002). Using data from the Human Epigenome Project (Eckhardt et al. 2006), we confirmed that hypomethylation in sperm corresponded to high values of the CpG o/e outside CGIs (data not shown). We also observed that CGIs' majoritary hypomethylated state spreaded out 1 kbp around the annotated CGIs, so that the sequence coverage by CGIs enlarged 1 kbp at both extremities provided a complementary marker for hypomethylated regions (Audit et al. 2009). When averaging over the 663 N-domains, the overall 1-kbp-enlarged CGI coverage (Fig. 3.4c) presents a maximum at origins positions, as the signature of hypomethylation, and decreases over a characteristic distance ~150 kbp, similar to the one found for DNase I HS coverage and NFR density profiles (Fig. 3.4a, b, d), from the extremities to the center of N-domains where CGI coverage is ~5 times less than the genome average. This observation is consistent with the hypothesis (Antequera and Bird 1999) that CGIs are protected from methylation due to there co-localisation with replication origins. The complementary analysis using the CpG o/e ratio as hypomethylation marker provided exactly the same diagnosis (data no shown).

N-domain borders correspond to a high concentration of genes (see below) (Huvet et al. 2007), TSS density profiles presenting, as expected, a strong similarity with 1-kbp-enlarged CGI coverage (Fig. 3.4c, e). Since the other open chromatin markers analyzed here have also been associated, at least to some extent, with genes (e.g. in $CD4^+$ cells, 16% of all DNase I HS are in the first exon or at the TSS of a gene, and 42% are found inside a gene (Boyle et al. 2008)), we reproduced the analysis of their distribution along the N-domains after masking the genes extended by 2 kbp at both extremities and the CGIs. The fact that the CpG o/e profiles (Fig. 3.4c), as well as the mean DNase I HS coverage and NFR density (data not shown) still present the decaying behavior over ~150 kbp, demonstrates that the excess observed around the putative replication origins does not simply reflects the rather packed gene organization at the N-domain borders but more likely an hypomethylated open chromatin state where CpG o/e is correlated with DNase I HS coverage and NFR density (Audit et al. 2009).

3.4.4 Coordination of Replication and Transcription at N-domain Borders

In higher-eukaryotes, extensive connections have been established between replication timing, genome organization and gene transcriptional state; early replication tends to co-localize with active transcription and, in mammals, to gene-dense GC rich isochores (Goldman et al. 1984; Schübeler et al. 2002; MacAlpine et al. 2004; Woodfine et al. 2005; Farkash-Amar et al. 2008; Hiratani et al. 2008; Karnani et al. 2007) and to transcription initiation early in development (Sequeira-Mendes et al. 2009). Correspondingly, N-domain borders are at the heart of a remarkable gene organization (Huvet et al. 2007): in a close neighborhood, genes are abundant and broadly expressed and their transcription is mainly directed away from the

borders (data not shown). All these features weaken progressively with the distance to domain borders. This prefential orientation was interpreted as a gene coorientation with the movement of replication fork originating from N-domain borders (Huvet et al. 2007).

CGIs were linked to the presence of a promoter active in the germline in a single-gene analysis in mouse (Macleod et al. 1998) and genes with a CGI associated promoter where shown to be expressed in early embryo using a dataset of ~400 human genes with a mouse ortholog (Ponger et al. 2001). Hence, we used CpG enrichment at the promoter as an indicator of germline expression. We observed that the distribution of CpG-rich promoters presents a strong enrichment at N-domains borders, whereas the density of CpG-poor genes does not present such a dependence with the distance to N-domain border. The preference for N-domain border proximity is thus specific to the set of genes more likely to be expressed in the germline, indicating that regions around N-domain borders are likely to be transcriptionally active in the germline. Thus the open chromatin regions around N-domain borders are permissive to transcription whereas N-domain central regions appear transcriptionally quiescent and further illustrates the likely coordination of replication and transcription at N-domain borders in the germline.

3.4.5 Open Chromatin Around N-domain Borders are Potentially Fragile Regions Involved in Chromosome Instability

Since chromatin accessibility and openness are possible factors responsible for fragility and instability, N-domain borders could also play a key role in genome dynamics during evolution. and genome instability in pathologic situations like cancer. We analyzed the distribution of evolutionary breakpoint regions for the mammalian lineage (Lemaitre et al. 2008) along N-domains. We observed that breakpoints appear more frequently near N-domain borders than in their central regions (Fig. 3.4f), suggesting that the distribution of large-scale rearrangements in mammals reflects a mutational bias towards regions of high transcriptional activity and replication initiation (Lemaitre et al. 2009). Furthermore, the fact that chromosome anomalies involved in the tumoral process like at the RUNX1T1 oncogene locus coincide with replication N-domain extremities (data not shown) raises the possibility that the replication origins detected *in silico* are potential candidate loci susceptible to breakage in some cancer cell types.

3.5 Conclusion

To summarize, we have found that replication associated mutational asymmetry is responsible for Mbp sized N-shaped domains along the nucleotide compositional asymmetry profiles of human chromosomes. We systematically detected these

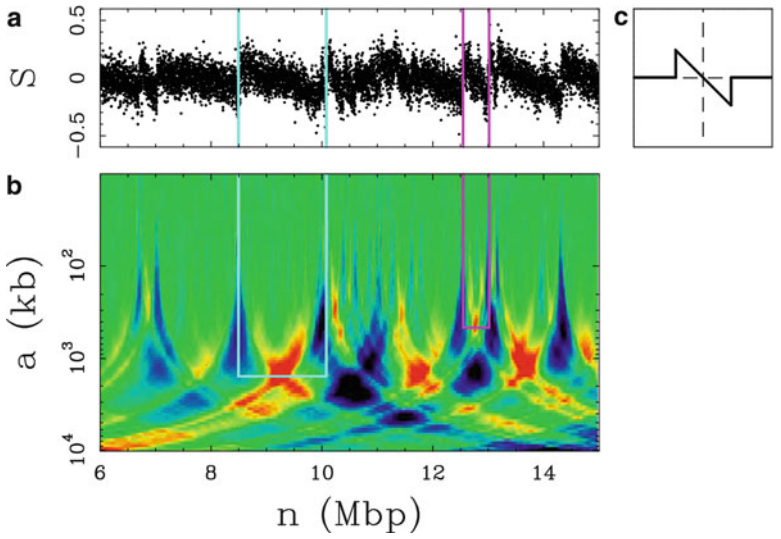

Fig. 3.5 Multi-scale detection of replication skew N-domains. (**a**) Skew profile *S* of a 9 Mbp repeat-masked fragment of human chromosome 21. (**b**) WT of *S* using the N-shaped wavelet illustrated in (**c**); the WT is color-coded from *dark-blue* (min; negative values) to *red* (max; positive values) through *green* (null values). *Light-blue* and purple lines illustrate the detection of two replication domains of significantly different sizes. Note that in (b), *blue* cone-shape areas signing upward jumps point at small scale (*top*) towards the putative replication origins and that the vertical positions of the WT maxima (*red areas*) corresponding to the two indicated replication domains match the distance between the putative replication origins (1.6 Mbp and 470 kbp respectively)

replication N-domains using an adapted wavelet-based methodology (Fig. 3.5). The 663 N-domains cover ~1/3 of the human genome and are bordered by 1,040 putative replication origins of the germline. These origins significantly overlap with experimental replication origins determined over ENCODE pilot regions (1% of the human genome) (Table 3.1). We developed a multi-scale analysis of high-resolution replication timing profiles allowing us to characterize in 5 cell lines preferential replication initiation zones as peaks pointing towards early time (Fig. 3.6). The analysis of the co-localization between N-domain borders and these timing peaks provides further genome-wide experimental evidence that N-domain borders are certainly preferential replication initiation zones mostly active in the early S phase, whereas N-domain central regions replicated more likely in late S phase (Table 3.2 and Fig. 3.2). These experimental verifications of *in silico* replication origin predictions are even more convincing when considering that, on top of the limitations due to experimental resolution, putative origin predictions concern only the replication origins that are well positioned and active in germline cells which does not guaranty that they are also active in somatic cells. Reciprocally, there is no guaranty that the replication origins that are active in this particular cell line are also active in the germline. In that respect, the results reported in this work provide interesting estimate of the proportion of preferential replication initiation

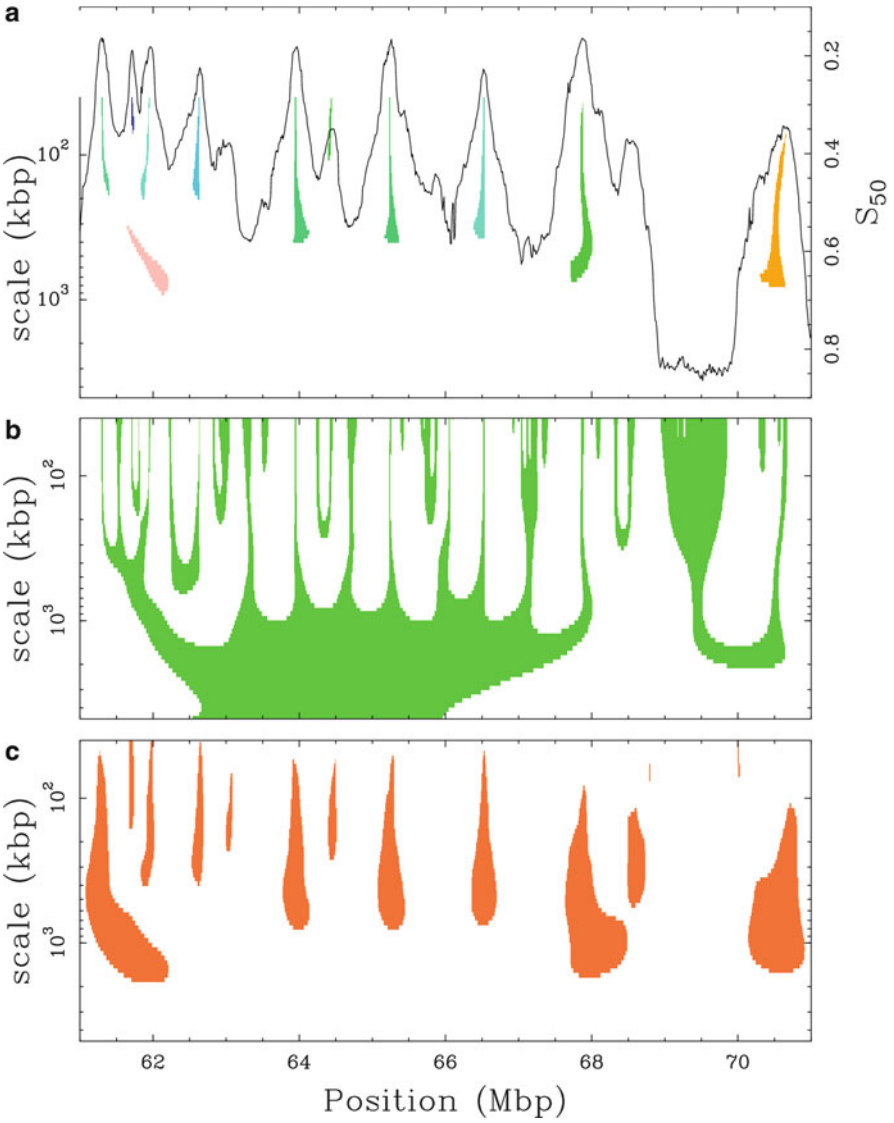

Fig. 3.6 (**a**) Timing profile normalised between 0 (start of S phase) and 1 (end of S phase). The color patches are space-scale representations of the detected peaks (see main text). (**b**) Regions of the space-scale half plane where the timing profile is flat according to: $\left|a^{-1/2}T_{g^{(1)}}[f](x,a)\right| < c_1$ with $c_1 = 0.01$. (**c**) Regions of the space-scale half plane where the timing profile present a significant negative curvature according to $a^{-1/2}T_{g^{(2)}}[f](x,a) < -c_2$ with $c_2 = 0.03$

zones that are active across several cell lineage (Fig. 3.3b). Our findings show that these replication initiation zones are located within a ~300 kbp region extremely sensitive to DNase I cleavage, presenting hypomethylation marks, suggesting that these regions present an open chromatin structure (Fig. 3.4a, b). This accessible chromatin organization is to some extent encoded in the DNA sequence via an enrichment of nucleosome excluding energy barriers (NFR) (Fig. 3.4d). The additional observation that the high gene density around N-domain borders is specific to CpG-rich TSS genes further suggests that this local chromatin structure is associated with transcriptional activity (Fig. 3.4e). The fact that experimental open chromatin signature obtained in a cell line appears specific to N-domain borders associated to timing peaks of the same cell line illustrates the correlation between replication activity and chromatin state. It raises the question of what are the determinants of this coordinated changes across cell differentiation.

In metazoans, recognition of replication origins by the origin recognition complex (ORC) does not involve simple consensus DNA sequence. Initiation sites do not share commmon genetic entities but seem to be favored by various factors that can differ from one origin to another and be required or dispensable under different conditions (Gilbert 2004). Specification of initiation sites can be favored by negatively supercoiled DNA (Remus et al. 2004) (possibly resulting from the removal or displacement of nucleosomes), by interacting proteins that chaperone ORC to specific chromatin sites (Schepers et al. 2001), by the transcriptional activity (Danis et al. 2004) or by open chromatin to which ORC might bind in a non-specific way (Vashee et al. 2003). A recent study performed on 283 replication origins identified in the ENCODE pilot regions showed that, besides a strong association with CGIs, only 29% overlap a DNase I hypersensitive site and that half of these origins do not present open chromatin epigenetic marks and are not associated with active transcription (Cadoret et al. 2008). The particular open chromatin state associated with N-domain borders suggest that these putative early replication origins present properties that are only shared by a subset of origins. These properties likely contribute to the specification of this peculiar subset of origins. The typical inter-origin distance in the human somatic cells has been estimated to be of the order of 50–100 kbp (Conti et al. 2007; Cadoret et al. 2008), a value significantly smaller than the typical size (1 Mbp) of N-domains. We propose that replication would initiate in early S phase at these privileged open chromatin locations and that the replication timing gradients observed from N-domain borders (Audit et al. 2007; Huvet et al. 2007; Baker et al. 2012) would correspond to the diverging replication forks progression triggering secondary origins in a “domino cascade” manner (Hyrien and Goldar 2010; Arneodo et al. 2011). As structural defects (bursts of “openness”) in the chromatin fiber, these replication initiation zones might also be central to the tertiary structure of eukaryotic chromatin into rosette-like structures (St-Jean et al. 2008). The present data suggest that they are likely to be associated with structuring chromatin elements playing an essential role in the spatio-temporal replication program.

3.6 Multi-scale Analysis of Compositional Asymmetry and Replication Timing Profiles

3.6.1 Delineating N-shaped Replication Domains Using Wavelets

The continuous wavelet transform (WT) is a space-scale analysis which consists in expanding signals in terms of wavelets that are constructed from a single function, the analyzing wavelet ψ, by means of dilations and translations (Muzy et al. 1994; Arneodo et al. 1995, 2002; Mallat 1998):

$$T_{\psi}[S](b,a) = \frac{1}{a}\int S(x)\psi\left(\frac{b-x}{a}\right)\mathrm{d}x. \tag{3.1}$$

where b and a (>0) are the space and scale parameters respectively. The wavelet coefficient $T_{\psi}[S](b,a)$ quantifies to which extent, around position b over a distance a, S has a similar shape as the analyzing wavelet ψ. Thus, using an adapted N-shaped analyzing wavelet constituted by a linearly decreasing segment between two upward jumps ($\psi(x) = -(x-1/2)$ for x $[0,1]$ and 0 elsewhere, Fig. 3.5c), we deploy the WT as an objective segmentation strategy of the human genome into candidate replication domains where the skew S displays a characteristic N-shaped pattern (Figs. 3.1b and 3.2a) (Audit et al. 2007; Huvet et al. 2007; Baker et al. 2010). The space-scale location of significant maxima values in the 2d WT decomposition (red areas in Fig. 3.5b) indicates the middle position (spatial location) of candidate replication domains whose size is given by the scale location. In order to avoid false positives, we then check that there does exist a well-defined upward jump at each domain extremity. These jumps appear in Fig. 3.5b as blue cone-shape areas pointing at small scale to the upward jumps positions where are located the putative replication origins.

But, the overall observed skew S also contains some contribution induced by transcription that generates step-like blocks corresponding to (+) and (−) genes (Touchon et al. 2003, 2004; Huvet et al. 2007; Nicolay et al. 2007). Hence, when superimposing the replication N-shaped and transcription step-like skew profiles, we get the following theoretical skew profile in a replication domain (Audit et al. 2007; Baker et al. 2010):

$$S(x') = S_R(x') + S_T(x') = -2\delta \times \left(x' - 1/2\right) + \sum_{\text{gene}} c_g \chi_g(x'), \tag{3.2}$$

where position x' within the domain has been rescaled between 0 and 1, $\delta > 0$ is the replication bias, χ_g is the characteristic function for the gth gene (1 when x' points within the gene and 0 elsewhere) and c_g is its transcriptional bias calculated on the Watson strand (likely to be positive for (+) genes and negative for (−) genes). The objective is thus to detect human replication domains by delineating, in the noisy S profile obtained at 1 kbp resolution (Fig. 3.2a), all chromosomal loci where S is well fitted by the theoretical skew profile Eq. 3.2. In order to enforce strong

compatibility with the mammalian replicon model (Sect. 3.2), we only retain the domains the most likely to be bordered by putative replication origins, namely those that are delimited by upward jumps corresponding to a transition from a negative S value $< -3\%$ to a positive S value $> +3\%$. Also, for each domain so-identified, we use a least-square fitting procedure to estimate the replication bias δ, and each of the gene transcription bias c_g. The resulting χ^2 value is then used to select the candidate domains where the noisy S profile is well described by Eq. 3.2.

3.6.2 *Multiscale Detection of Peaks in Replication Timing Profiles*

The simple intuitive idea allowing for effective detection of peaks in a noisy profile f is to delineate positions x along the signal that are a local extrema ($f'(x) \sim 0$) and present a strong (negative) curvature ($f''(x) \ll 0$) as expected at the tip of a peak symmetrical about a vertical axis. In order to avoid the confusion between "true" peaks and those induced by the presence of a noisy background, the rates of signal variation have to be estimated over a sufficiently large number of data points. This can be achieved using the continuous wavelet transform (WT) which provides a powerful framework for the estimation of signal variations over different length scales (Mallat 1998; Arneodo et al. 2002).

When using as the analyzing wavelet the derivatives of the Gaussian function, namely $g^{(n)}(x) = \mathrm{d}^n g^{(0)}(x) / \mathrm{d}x^n$, with $g^{(0)}(x) = \frac{1}{\sqrt{2\pi}} e^{-x^2/2}$, then the WT of a profile f takes the following expression:

$$T_{g^{(n)}}[f](x,a) = a^n \frac{\mathrm{d}^n}{\mathrm{d}x^n} (g_a^{(0)} * f)(x), \tag{3.3}$$

where x and a (>0) are the space and scale parameters respectively. Equation 3.3 shows that the WT computed with $g^{(n)}$ is proportional to the n^{th} derivative of the profile f smoothed by a dilated version $g_a^{(0)}(x) = \frac{1}{a} g^{(0)}(x/a)$ of the Gaussian function. This property is at the heart of various applications of the WT microscope as a very efficient multi-scale singularity tracking technique (Mallat 1998; Arneodo et al. 2002). When the profile of f is the graph of a Brownian motion i.e. the increments of f are independent, identically distributed Gaussian variables, then the WT at scale a is Gaussian with a standard deviation proportional to $a^{1/2}$. Hence, the amplitude of the fluctuations of $a^{-1/2} T_g^{(n)}[f](x,a) \sim \mathcal{N}(0, \sigma_o)$ are independent of the scale of analysis.

The basic principle of the detection of peaks in the replication timing profiles with the WT is illustrated in Fig. 3.6. In a first step, we determine (i) the regions of the space-scale half plane candidates to be a local extrema applying the following thresholding of the WT of f using $g^{(1)}$: $|a^{-1/2} T_g^{(1)}[f](x,a)| < c_1$ (Fig. 3.6b) and (ii) the regions of strong concavity applying the following thresholding of the WT of f using $g^{(2)}$: $a^{-1/2} T_g^{(2)}[f](x,a) < -c_2$ (Fig. 3.6c). In this way, both thresholds on the first

and second derivatives are uniform with respect to the fluctuations for a Brownian profile. In a second step, we determine the connected regions of the space-scale half plane where both requirements are fulfilled (color regions in Fig. 3.6a). Finally, connected regions that have a scale extension (ratio between the region largest and smallest scales) smaller than 1.74 are disregarded in order to guaranty the existence of a well defined peak robust with respect to the scale of observation.

3.7 Material

3.7.1 Sequence and Annotation Data

Sequence and annotation data were retrieved from the Genome Browsers of the University of California Santa Cruz (UCSC) (Karolchik et al. 2003). Analyses were performed using the human genome assembly of March 2006 (NCBI36 or hg18). As human gene coordinates, we used the UCSC Known Genes table. When several genes presenting the same orientation overlapped, they were merged into one gene whose coordinates corresponded to the union of all the overlapping gene coordinates, resulting in 23,818 distinct genes. We used CpG islands (CGIs) annotation provided in UCSC table "cpgIslandExt".

3.7.2 CpG Observed/Expected Ratio

CpG observed/expected ratio (CpG o/e) was computed as $\frac{n_{CpG}}{L-l} \times \frac{L^2}{n_C n_G}$, where n_C, n_G and n_{CpG} are the number of C, G and dinucleotides CG counted along the sequence, L is the number of non-masked nucleotides of the sequence and l the number of masked nucleotide gaps plus one, i.e., $L-l$ is the number of dinucleotide sites.

3.7.3 Determining Mean Replication Timing Profiles

We determined the mean replication timing profiles along the complete human genome using Repli-Seq data (Hansen et al. 2010; Chen et al. 2010). For ESC cell line (BG02), a lymphoblastoid cell lines (GM06990), a fibroblast cell line (BJ), and erythroid K562 cell line, Repli-Seq tags for 6 FACS fractions were obtained directly from the authors (Hansen et al. 2010). For the HeLa cell line we computed the mean replication timing (MRT) instead of computing the S50 (median replication timing) as in (Chen et al. 2010).

3.7.4 DNase I Hypersensitive Site Data

We used the DNase I sensitivity measured genome-wide (Sabo et al. 2006). Data corresponding to Release 3 (Jan 2010) of the ENCODE UW DNaseI HS track, were downloaded from the UCSC FTP site: ftp://hgdownload.cse.ucsc.edu/goldenPath/hg18/encodeDCC/wgEncodeUwDnaseSeq/.

We plotted the coverage by DNase I hypersentive sites identified as signal peaks at a false discovery rate threshold of 0.5% within hypersensitive zones delineated using the HotSpot algorithm ("wgEncodeUwDnaseSeqPeaks" tables). When several replicates were available, data were merged.

3.7.5 Genome-Wide Nucleosome Positioning Data

We used the genome-wide map of nucleosome positioning in resting human $CD4^+$ T cells obtained from direct sequencing of nucleosome ends using the Solexa high-throughput sequencing technique (Schones et al. 2008). Nucleosome score profiles for human genome assembly hg18 were downloaded from http://dir.nhlbi.nih.gov/papers/lmi/epigenomes/hgtcellnucleosomes.html.

3.7.6 CpG-Rich and CpG-Poor Promoters

We noticed that several promoters that were not overlapping a CGI yet had an enrichment in CpG content at the promoter and displayed the same characteristics in terms of Pol II binding and H3K4me3 enrichment as genes with a CGI associated promoter. Hence following previous work (Saxonov et al. 2006), we considered the CpG o/e at the promoter calculated in a 1 kbp window centered on the TSS rather than its association to a tabulated CGI, whose definition is based on *ad-hoc* criteria. Thanks to a clear bimodal distribution of CpG o/e at the promoters, we could objectively separate genes based on CpG-rich (CpG o/e >0.48) and CpG-poor (CpG o/e <0.48) promoters. According to this criteria 65% of the genes are CpG-rich.

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Chapter 4
Role of DNA Methyltransferases in Epigenetic Regulation in Bacteria

Ritesh Kumar and Desirazu N. Rao

Abstract In prokaryotes, alteration in gene expression was observed with the modification of DNA, especially DNA methylation. Such changes are inherited from generation to generation with no alterations in the DNA sequence and represent the epigenetic signal in prokaryotes. DNA methyltransferases are enzymes involved in DNA modification and thus in epigenetic regulation of gene expression. DNA methylation not only affects the thermodynamic stability of DNA, but also changes its curvature. Methylation of specific residues on DNA can affect the protein-DNA interactions. DNA methylation in prokaryotes regulates a number of physiological processes in the bacterial cell including transcription, DNA mismatch repair and replication initiation. Significantly, many reports have suggested a role of DNA methylation in regulating the expression of a number of genes in virulence and pathogenesis thus, making DNA methlytransferases novel targets for the designing of therapeutics. Here, we summarize the current knowledge about the influence of DNA methylation on gene regulation in different bacteria, and on bacterial virulence.

4.1 Introduction

DNA methylation is known to play a critical role in epigenetic gene regulation in prokaryotes and eukaryotes (Wion and Casadesus 2006; Marinus and Casadesus 2009). While it is N^6 adenine methylation which brings about the epigenetic control in bacteria, it is methylation of cytosine at C^5 position in eukaryotes (Marinus and Casadesus 2009; Bestor 2000). The microenvironment in which bacteria grows is highly dynamic. Epigenetic regulation of gene expression helps the bacteria to cope up with the changing environment. Bacteria respond to changes in the environment,

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such as nutrient availability, temperature, pH, osmolarity by regulating gene expression (Wion and Casadesus 2006). A regulated expression of genes according to stimuli is critical for successful survival. For pathogenic bacteria it is very essential to cope up with host immune response and to colonize different microenvironments and regulated expression of some critical genes plays a vital role in enhancing the adaptability.

It has been known for a long time that DNA in different organisms has methylated bases N^6- methyladenine, C^5 methylcytosine and N^4 methylcytosine (Jeltsch 2002). All the three modified bases could be found in bacteria but eukaryotes are known to have only C^5 methylcytosine (Jeltsch 2002). Methylation of the bases does not affect the Watson/Crick pairing properties of adenine and cytosine. However, addition of the methyl group can be easily detected by proteins interacting with the DNA. Thus, methylation adds extra information on DNA without changing the sequence (Wion and Casadesus 2006).

It has been shown that C^5 cytosine methylation in eukaryotes plays significant role in epigenetic regulation (Nan et al. 1998; Jorgensen and Bird 2002; Klose and Bird 2006). Methylation of CG sites by eukaryotic DNA methyltransferases is involved in gene regulation. A number of genes in eukaryotes are known to have CG sites in their promoter region and the methylation status of CG sites affects the gene expression (Lewis et al. 1992; Klose and Bird 2006). Methylation of CG sites not only abrogates the binding of number of transcription factors, but methylated CG sites also recruit 5-methylcytosine binding proteins, which represses transcription (Nan et al. 1998). In prokaryotes it is the N^6 methyl adenine which is known to affect the DNA protein interaction and thus, affecting the gene expression (Casadesu's and Low 2006). DNA adenine methyltransferase (Dam) is the most studied methyltransferase involved in epigenetic regulation in bacteria. Dam recognizes 5′ GATC 3′ and methylates adenine on both strands (Fälker et al. 2007). It is the hemimethylated form of DNA formed just after the DNA replication of a fully methylated DNA, which acts as a signal by different DNA interacting proteins. For example, in case of replication it is SeqA protein which interacts with GATC sites in the hemimethylated DNA clustered in the origin of replication (*oriC*) and checks the replication initiation (Braun et al. 1985; Yamaki et al. 1988). Methyl directed mismatch repair protein MutH interacts with GATC sites in hemimethylated DNA and cuts the unmethylated strand to ensure that methylated strand is used as a parental strand for repair-associated DNA synthesis (Marinus 1996). Cell cycle regulated methyltransferase (CcrM) is another well studied DNA methyltransferase involved in gene regulation (Reisenauer et al. 1999; Reisenauer and Shapiro 2002). Sequencing of *Helicobacter pylori* strains have unveiled that *H. pylori* genome is rich in DNA methyltransferases associated with restriction enzymes. They also contain a number of solitary methyltransferases different from Dam and CcrM (McClain et al. 2009; Vitkute et al. 2001; Tomb et al. 1997). Recent findings have shown that methyltransferases associated with R-M systems can affect the gene expression possibly by promoter methylation (Srikhanta et al. 2005). A number of methyltransferases associated with differential gene expression are components of Type III R-M systems and few of them exhibit phase variation thus, adding an extra dimension in the gene regulation in bacteria (Srikhanta et al. 2005, 2010).

4.2 Restriction-Modification System in Bacteria

DNA methylation was discovered in the context of restriction- modification system (R-M). R-M systems were discovered as the consequence of observations made in the early 1950s on host-controlled variation of bacterial viruses (Luria and Human 1952; Bertani and Weigle 1953). Prokaryotic restriction-modification (R-M) systems were first recognized in *Escherichia coli* nearly 50 years ago (Arber and Dussoix 1962) and are now known to be ubiquitous among bacterial species. In general, R-M systems consist of two components having distinct enzymatic activities: first, a restriction endonuclease that cleaves DNA at a specific recognition sequence, and second, a DNA methyltransferase that methylates DNA at the same site and thus prevents cleavage by the cognate restriction enzyme (Raleigh and Brooks 1998). Restriction endonucleases occur ubiquitously among bacteria, archaea (Bickle and Kruger 1993; Raleigh and Brooks 1998; Roberts et al. 2003, 2007; Sistla and Rao 2004; Pingoud et al. 2005), in viruses of certain unicellular algae (Van Etten 2003), and they are usually accompanied by a modification enzyme of identical specificity. Together, the two activities form a R-M system- the prokaryotic equivalent of an immune system. Their main function is to protect their host genome against foreign DNA. The host DNA is resistant to cleavage, as these sites are modified by cognate methyltransferase. It was proposed that R-M system has functions other than host protection, including maintenance of species identity among bacteria (Jeltsch 2003) and generation of genetic variation (Arber 2000, 2002). Restriction endonucleases of *Chlorella* viruses may have a nutritive function by helping in degradation of host DNA or preventing infection of a cell by another virus (Van Etten 2003). Other functions have also been suggested, such as involvement in recombination and transposition (Carlson and Kosturko 1998; Heitman 1993; McKane and Milkman 1995). Many R-M systems can also be considered as selfish DNA elements (Naito et al. 1995; Kobayashi 2004). In general, bacteria and archaea harbour multiple types of R-M systems. For example, in *H. pylori* more than 20 putative R-M systems, comprising greater than 4% of the total genome, have been identified in two completely sequenced *H. pylori* strains (Lin et al. 2001). These enzymes represent the largest family of functionally related enzymes. Based on the number and organization of subunits, regulation of their expression, cofactor requirements, catalytic mechanism, and sequence specificity, restriction enzymes have been classified into four different types. The broader classification includes Types I, II, III, and IV (Wilson and Murray 1991; Roberts et al. 2003, 2007; Sistla and Rao 2004; Pingoud et al. 2005).

4.2.1 DNA Methyltransferases

Post-replicative base methylation is the most common DNA modification in bacteria. C^5-methylcytosine and N^6-methyladenine are found in the genomes of many

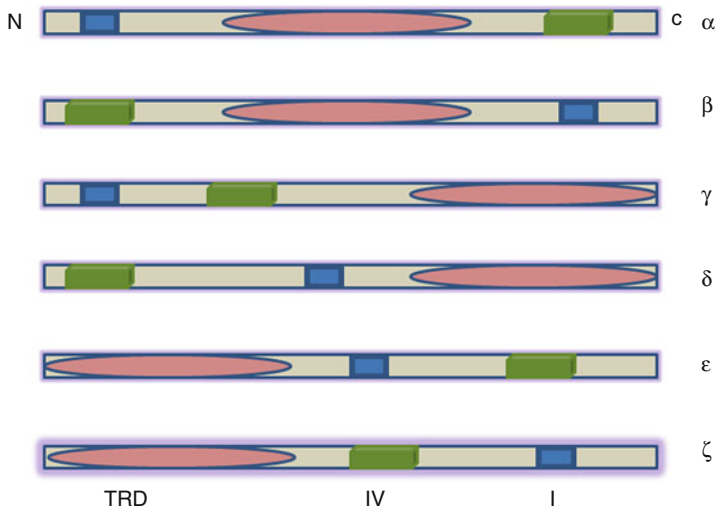

Fig. 4.1 Schematic diagram representing the arrangement of conserved motifs in the primary structure of DNA MTases. The linear arrangements of three consecutive motifs, AdoMet-binding domain, motif I; the catalytic domain, motif IV; the TRD, target recognition domain are shown

fungi, bacteria and protists, whereas N^4-methyl-cytosine is found only in bacteria (Cheng 1995). N^6-methyl-adenine is also present in archaeal DNA (Barbeyron et al. 1984; Cheng 1995). Two classes of DNA methyltransferases perform base modifications in bacterial genomes: those associated with R–M systems (Bickle and Kruger 1993), and solitary methyltransferases that do not have a restriction-enzyme counterpart. Based on the chemistry of the methylation reaction catalyzed, DNA methyltransferases are classified as endocyclic methyltransferases, which transfer the methyl group from AdoMet to C^5 position of cytosine (^{m5}C), and exocyclic amino methyltransferases, which transfer the methyl group to the exocyclic amino group of adenine (^{m6}A) or cytosine (^{m4}C), respectively (Bheemanaik et al. 2006). DNA methyltransferases of all three types contain conserved regions, which are responsible for catalysis and AdoMet binding, and variable regions known as target recognition domains (TRD), which determine the substrate specificity of a particular enzyme. Ten conserved amino acid motifs (I–X) were found in C^5 methyltransferases, the motif order being always constant (Posfai et al. 1989; Kumar et al. 1994). DNA methyltransferases are subdivided further into six groups (namely α, β, γ, ζ, δ and ε) (Fig. 4.1), according to the linear arrangements of three conserved motifs, the AdoMet-binding domain (FXGXG), the TRD (target recognition domain) and the catalytic domain (D/N/S)PP(Y/F) motifs (Malone et al. 1995; Bheemanaik et al. 2006).

4.2.2 Orphan DNA Methyltransferases

Most of the DNA methyltransferases known are the component of R-M systems. However, some of the methyltransferases lack cognate restriction enzymes (Casadesu's and Low 2006). They are termed as orphan or solitary methyltransferases. Solitary methyltransferases include the N^6-adenine methyltransferases Dam (**D**NA **a**denine **m**ethyltransferase) and CcrM (**c**ell **c**ycle- **r**egulated **m**ethyltransferase), and the Dcm (**D**NA **c**ytosine **m**ethyltransferase) (Marinus 1996; Reisenauer et al. 1999; Low et al. 2001; Lobner-Olesen et al. 2005; Wion and Casadesus 2006; Marinus and Casadesus 2009).

E. coli DNA adenine methyltransferase (EcoDam) recognizes the sequence 5′-GATC-3′ and methylates at the N^6 position of the adenine (Geier and Modrich 1979). EcoDam showed preference towards the *in vivo* substrate hemimethylated DNA (Herman and Modrich 1982). EcoDam was able to methylate denatured DNA substrates and single stranded synthetic oligonucleotides with lesser rates. Methylation of the GATC sequence was shown to be modulated by the nature of the three base pairs flanking both sides of the site (Bergerat et al. 1989). However, it was demonstrated using short, synthetic duplexes, that EcoDam does not prefer hemimethylated DNA substrates to unmethylated substrates (Bergerat et al. 1989).

It was observed that a functional monomeric EcoDam methylates only one strand of the DNA in each binding event (Urig et al. 2002). They showed that EcoDam scans 3000 GATC sites per binding event randomly and methylates GATC sites on DNA processively. On the contrary, Mashhoon et al. (2004) demonstrated that *E. coli* Dam methylates adjacent GATC sites in a distributive manner and thus controls the transcription of the adjacent genes.

T4 DNA adenine methyltransferase (T4Dam) from bacteriophage T4, recognizes the sequence 5′-GATC-3′ and methylates the adenine residue at the N^6 position (Schlagman and Hattman 1983; Hattman and Malygin 2004). It is capable of methylating unmethylated, hemimethylated GATC sequences and also methylates noncanonical sites but less efficiently (Schlagman et al. 1988; Kossykh et al. 1995). Cell-cycle-regulated methyltransferase (CcrM) is another N^6 adenine methyltransferase without a cognate restriction enzyme. CcrM has been identified in number of bacterial species and it recognizes the sequence 5′ GANTC 3′ and methylates adenine (Zweiger et al. 1994). Unlike Dam which belongs to γ -group, CcrM belongs to β-group of DNA methyltransferases, and shares homology with HinfI methylase of *Haemophilus influenzae* (Reisenauer et al. 1999).

Dcm is another orphan methyltransferase which methylates cytosine at C^5 position. The product of the *dcm* gene is the only DNA C^5-cytosine methyltransferase of *E. coil* K-12 which catalyses transfer of a methyl group to the C^5 position of the inner cytosine residue of the cognate sequence CCA/TGG. Till date there are no reports on the role of cytosine methylation in epigenetic regulation. However it has been shown that DNA cytosine- methyltransferase (Dcm) is associated with very short repair (*vsr*) endonuclease (Sohail et al. 1990).

4.3 DNA Methylation Dependent Regulatory Systems

Methylation of the specific DNA sequences by solitary methyltransferases is involved in bacterial physiology, pathogenesis, and host–pathogen interactions. It was proposed that N^6-methyl-adenine can act as an epigenetic signal for DNA–protein interactions in bacteria, much similar way as C^5-methylcytosine in eukaryotes (Wion and Casadesus 2006). Dam methylation provides signals for DNA protein interactions (Polaczek et al. 1998; Messer and Noyer-Weidner 1988). Dam is found to be involved in many of the vital cellular processes: Chromosome replication and nucleoid segregation, Dam-directed mismatch repair, regulation of transposition, phase variation, bacteriophage infection and bacterial conjugation (Fig. 4.2) (Marinus 1996; Reisenauer et al. 1999; Low et al. 2001; Lobner-Olesen et al. 2005).

Transposition of insertion sequences play an important role in the biology of bacteria. It has been shown that the methylation pattern near or in the transposon sequences control the rate of transposition (Dodson and Berg 1989; Reznikoff 1993). In IS*10* promoter, a GATC site overlaps with −10 module and a second GATC site is present at the end of the transposon. Methylation of GATC site in the promoter hinders the binding of RNA polymerase and methylation of GATC at the end of transposon inhibits tansposase activity at these ends (Roberts et al. 1985). Similar to IS*10*, transposition of IS*50* and Tn*5* is also controlled by DNA methylation (McCommas and Syvanen 1988; Dodson and Berg 1989).

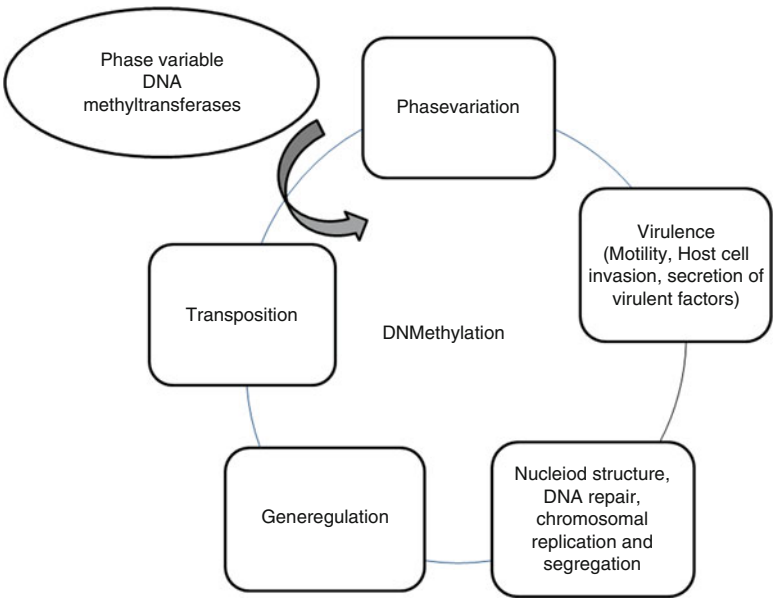

Fig. 4.2 Overview of the roles of N^6-methyl adenine in bacteria

A number of bacteriophages like T-even, PI, and Mu have *dam*, which methylate GATC sites in their genome (Blaisdell et al. 1996). It has been shown that methylation by Dam regulates the expression of *cre* gene of phage P1 and the *mom* gene of phage Mu (Sternberg et al. 1986; Hattman and Sun 1997). Dam methylation is also significant in packaging of P1 DNA into capsids. It has been shown that a P1 *dam*$^-$ mutant produces only 5% of the normal titre on infection of a *dam*$^-$ host (Yarmolinski and Sternberg 1988).

Dam methylation is important for the conjugal transfer of the virulence plasmid of *Salmonella enterica.* Dam methylation suppresses the transfer (*tra*) operon of the *Salmonella* virulence plasmid (pSLT) and F sex factor (Torreblanca et al. 1999). The effect of Dam methylation on *tra* operon is indirect. Dam methylation controls the expression of regulatory genes *traJ* and *finP*, which in turn affects the operon (Camacho and Casadesus 2002).

It was shown that Dam plays major role in virulence gene expression of *S. enterica* (Heithoff et al. 1999, 2001; Garcia-Del Portillo et al. 1999), *H. influenzae* (Watson et al. 2004), and *Yersinia pseudotuberculosis* (Taylor et al. 2005). Dam-overproducing strains of *Y. pseudotuberculosis* and *Yersinia enterocolitica* show increased secretion of Yops (Yersinia outer proteins), a group of virulence proteins that are involved in inhibition of phagocytosis and pro-inflammatory cytokine release (Julio et al. 2001, 2002; Fa¨lker et al. 2005). Overproduction of *E. coli* Dam methylase has also been shown to attenuate virulence in *Pasteurella multocida* (Chen et al. 2003). It was therefore proposed that Dam methylation might possibly regulate virulence gene expression in many other pathogens. It has been reported that mutation of the solitary N^6 adenine methyltransferase Cj1461 affects several phenotypes related to virulence in *Campylobacter jejuni*, suggesting that epigenetic regulation may play a role in *C. jejuni* pathogenesis (Kim et al. 2008). CcrM, a solitary methyltranferase is essential and participates in cell-cycle regulation in *Caulobacter crescentus, Brucella*, *Agrobacterium* and *Sinorhizobium*, (Reisenauer and Shapiro 2002; Zweiger et al. 1994; Marczynski and Shapiro 2002; Robertson et al. 2000). However, no biological function has yet been demonstrated for Dcm (Wion and Casadesus 2006). *E. coli* very short patch (*vsr*) gene is in a transcriptional unit with *dcm*. Intriguingly, deletion of *dam* in *E. coli* reduces VSP repair (Bell and Cupples 2001).

4.3.1 DNA Methylation: Critical for Replication and Repair

Initiation of DNA replication is highly regulated process in bacteria and DNA methylation plays a significant role in regulating the initiation process. In a number of bacteria it has been shown that Dam methylation is important for regulation of a number of processes, like replication and repair (Casadesu's and Low 2006). DnaA protein initiates the replication by binding to *oriC*. OriC and promoter of *dnaA* is rich in GATC sequence and the methylation status of these GATC sites controls the initiation of replication (Bogan and Helmstetter 1997; Campbell and Kleckner 1990). A first round of replication results in hemimethylated DNA and hemimethylated DNA

acts as a binding site for SeqA protein. Sequestration by SeqA prevents the reinitiation of replication more than once per cell cycle (Lu et al. 1994). For an efficient initiation, *oriC* and *dnaA* promoter should be fully methylated. DNA methylation is therefore, not essential for DNA replication but regulates the timing of replication initiation. Hemimethylated state of *oriC* acts as a signal for nucleoid segregation. The hemimethylated origin of replication of both daughter chromosomes bind to proteins involved in segregation (Ogden et al. 1988). During DNA replication, DNA polymerase can incorporate wrong bases and this results in mismatch bases in DNA. DNA mismatch repair system corrects the base mismatches that can occur during the synthesis of new unmethylated strand (Au et al. 1992). DNA methylation by Dam acts as a marker for the repair machinery to distinguish between methylated parental strand and unmethylated newly synthesized strand. Any alteration in expression of Dam in bacterial cell (increase or decrease in Dam concentration in the cell) results in a mutator phenotype (Løbner-Olesen et al. 2003; Calmann and Marinus 2003; Oshima et al. 2002; Julio et al. 2002). Deletion of *dam* in bacterial cell results in the loss of strand discrimination by mismatch repair proteins, resulting in the use of parental strand as a template for repair with a 50% probability (Modrich and Lahue 1996; Chen et al. 2003). On the other hand, overproduction of Dam leads to premature methylation of newly synthesised strand, therefore preventing MutH action on a mismatch (Marinus 1996). It must be noted that Dam expression is tightly regulated in cell at the transcriptional level, involves multiple promoters, and is growth rate regulated (Marinus 1996).

4.3.2 Regulation of Gene Expression

DNA methylation can affect gene expression in two ways. First, methylation of cognate recognition sequences in the promoter regions can increase, decrease, or have no change in transcription initiation by affecting the interaction between RNA polymerase and promoters. A second mechanism involves the competition between Dam and regulatory proteins (Cap, Lrp or OxyR) for overlapping sites in or near promoters/ regulatory regions on DNA (Low et al. 2001). A number of studies with the knockout strains of *dam* have shown that the *dam* deletion affects the transcript levels of a number of genes involved in different pathways such as motility, virulence, lipid and aminoacid metabolism and cofactor biosynthesis (Table 4.1) (Heithoff et al. 2001; Oshima et al. 2002; Watson et al. 2004).

The CcrM methyltransferase from *Caulobacter crescentus* plays an essential role in cell cycle. *C. crescentus* has two different cell forms: replicating stalked form and non-replicating swarmer form (Marczynski and Shapiro 2002). Cell division yields one motile swarmer cell and one non-motile stalked cell. CcrM is only produced only at the late stages of replication in the stalked cell (Reisenauer et al. 1999). Activation of Caulobacter replication origin (*Cori*) requires complete methylation, thus synthesis of CcrM at the later stages of DNA replication is the signature for competition of one cell cycle. Methylation of *Cori* by CcrM of alpha-proteobacteria

Table 4.1 Roles of DNA methyltransferases in the physiology of different bacterial species

Bacterial pathogen	Methylase	Recognition sequence	Effect of deletion	Effect of overexpression
Escherichia coli	Dam	GATC	Suppresses virulence, enhances mutation rates	Enhances the mutation rate
Caulobacter crescentus	CcrM	GANTC	Decreases viability	Defects in cell division and morphology
Brucella abortus	CcrM	GANTC	Decreases viability	Inhibits growth
Salmonella enterica	Dam	GATC	Attenuates virulence	
Vibrio cholerae	Dam	GATC	Essential for viability	Inhibits colonization in suckling mouse model
Yersinia pseudotuberculosis	Dam	GATC	Essential for viability	Attenuates virulence in murine model
Nesseria meningitidis	Dam	GATC	Enhances the rate of phase variation	
Haemophilus influenzae	Dam	GATC	Strain specific reduction in invasion and adhesion	
Yersinia enterocolitica	Dam	GATC	Essential in certain strains	Increase in motility, alters lipopolysaccharide O-antigen
Helicobacter pylori strain PG227	M.HpyAVIA	GAGG	Defects in growth	
Helicobacter pylori	M.HpyI	CATG	Alteration in *dnaK* stress-responsive operon expression	
Klebsiella pneumoniae	Dam	GATC	Partial attenuation in mice infection model	
Seromonas hydrophila	Dam	GATC	Necessary for viability	Defects in virulence
Campylobacter jejuni	CJ1461		Reduced motility and reduced invasion	
Haemophilus influenzae	Mod subunit of Type III R-M system (Phase variable)		Affects transcript levels of *dnaK* and several genes coding for surface proteins	
N. gonorrhoeae strain FA1090	ModA13	AGAAA	Five of the differentially regulated genes have roles in virulence; four in oxidative stress and one in antimicrobial resistance	
Edwardsiella tarda	Dam	GATC	Significant attenuation of overall bacterial virulence and altered several stress responses including spontaneous mutation, recovering from UV radiation and H_2O_2 exposure	

provides a signal for the initiation of next round of replication in these bacteria (Wright et al. 1996). Bacterial cells achieve this stringent control by regulating the concentration of CcrM and CtrA (a global regulator). CtrA controls the expression of CcrM and methylation by CcrM controls its own expression and CtrA expression. One of the two promoters of *ctrA* contains a GANTC site near −35 module (Marczynski and Shapiro 2002).

A number of adenine methyltransferases have been identified in *H. pylori* and it has been suggested that they could be playing significant roles in the gene expression regulation. M.*HpyI*, is M.*NlaIII* homolog present in *H. pylori* and is highly conserved among all *H. pylori* strains. It encodes a 329-amino-acid protein which recognizes DNA at sites containing CATG (Xu et al. 2000). The transcript levels of *hpyIM* expression vary with *in vitro* growth phase, with higher expression being noted during exponential growth than during stationary phase. Inactivation of *hpyIM* results in pleiotropic bacterial morphology including alteration in the expression of the *H.pylori dnaK* stress-responsive operon (Donahue et al. 2002). Recently, it has been shown in *H. pylori* strains PG227 and 128, deletion of a N^6 adenine methyltransferase M.HpyAVIA resulted in slow growth (Kumar et al. 2010).

4.3.3 DNA Methylation Affects the Host-Pathogen Interaction

Several reports suggest that various virulence-associated phenotypes are influenced by DNA methylation and DNA methylation targets regulatory processes modulating the composition and function of bacterial surface and thus, affecting the bacterial interaction with host (Heusipp et al. 2006). The involvement of DNA methylation in controlling the virulence was first described for *S. enterica* serovar Typhimurium. Deletion of Dam decreases the efficiency of the strain to colonize mice. *dam*⁻ strains of *Salmonella* are attenuated and present pleiotropic virulence-related defects including membrane instability, leakage of proteins, defect in motility and release of vesicles. *S. enterica* lacking Dam were effective in colonization of mucosal sites but showed several defects in colonization of deeper tissues (Heithoff et al. 1999; Oza et al. 2005; Heusipp et al. 2006). Deletion of *dam* in *H. influenzae* results in similar defects as observed in *S. enterica*, including decrease in invasion of host cells and decrease in virulence (Watson et al. 2004; López-Garrido and Casadesús 2010). In *Klebsiella pneumoniae*, a mutation eliminating Dam methylation has been shown to result in partial attenuation in a mouse infection model (Mehling et al. 2006). Dam mutants of *Yersinia pestis* and *Salmonella* serovar Typhimurium show 2,000-fold and 10,000-fold increase in lethal dose compared to wild type strain in orally infected mice (Heithoff et al. 1999; Julio et al. 2001; Robinson et al. 2005). It has been shown that natural population of *Neisseria* contains two biotype *dam*⁻ and *dam*⁺. Deletion of *dam* in one biotype results in high rates of phase variation in surface associated components and thus, affects its interaction with the host (Table 4.1) (Bucci et al. 1999). *Edwardsiella tarda* is a serious aquaculture pathogen and infects many fishes. It has been shown that deletion of *dam* in *E. tarada* alters

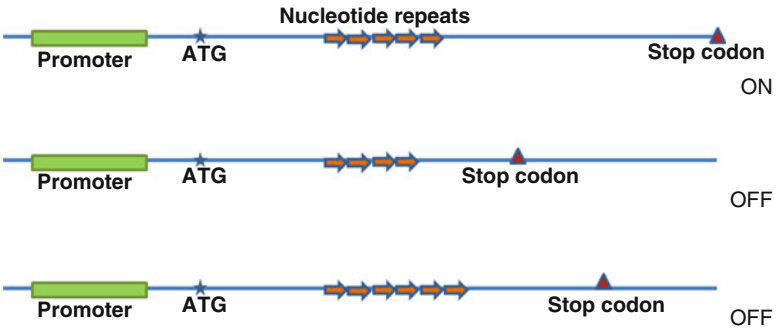

Fig. 4.3 Alteration in the number of repeats results in phase variation

the binding to host mucus (Sun et al. 2010) Deletion of *dam* erases the methylation pattern in the cells thus, affecting the number of protein- DNA interactions. It is not only the deletion of *dam* that affects the bacterial interaction with host but overexpression of Dam in *Vibrio cholera* and *P. multocida* have been shown to decrease virulence (Julio et al. 2001; Chen et al. 2003). Overexpression of Dam alters motility and invasiveness of *Y. enterocolitica* (Julio et al. 2001).

4.3.4 DNA Methylation and Phase Variation

A number of bacterial pathogens, such as *H. pylori*, *H. influenzae*, *Neisseria gonorrhoeae* and *Neisseria meningitidis* have evolved molecular mechanisms that can help them to adapt to the host. These organisms are capable of generating genetic variation, which in turn help them to cope up with the changing host environment and immune response. One common mechanism is phase variation, a reversible switch between an "all-or-none" (ON/OFF) expressing phase (Fig. 4.3), resulting in variation in the level of expression of one or more proteins between individual cells of a clonal population (Weiser et al. 1990; van Ham et al. 1993; Saunders et al. 1998; Hallet 2001). Phase variation results in two sub populations in a clonal population: one lacking or having a decreased level of expression of a phase variable gene and other population with full expression of the gene. The classical view of phase variation and antigenic variation is that its role is to help the bacterium evade the host immune system. This is supported by the fact that most of the genes that are subjected to phase variation are surface antigens such as lipopolysaccharide (LPS) and outer-membrane proteins (OMPs) where they would be exposed to the immune system (Saunders et al. 1998; Jennings et al. 1995; van der Ende et al. 1995) and play a vital role in bacteria and host interaction. Phase variation, therefore helps the pathogen in colonization of the host, adaptation to the dynamic host environment and evasion of immune responses (Moxon et al. 2006). Phase variation can occur through several mechanisms like (i) Short sequence repeats and slipped-strand mispairing (SSM) mechanisms (ii) Homologous (general) recombination

(iii) Site-specific recombination (iv) Environmental regulation and (v) Epigenetic Regulation (Moxon and Thaler 1997; Robertson and Meyer 1992; van der Woude and Baumler 2004; van der Woude 2006). In contrast to the other mechanisms mentioned above, epigenetic regulation of phase variation occurs in the absence of a change in DNA sequence. In epigenetic regulation, the phenotypes are altered but the genotypes are not. DNA methylation plays a critical role in this type of phase variation system, as the methylation state of a target sequence at a specific site in the chromosome affects the DNA binding of a regulatory protein that directly regulates transcription. *E.coli* and *S.entrica* serotype Typhimurium exhibit methylation-dependent phase variation (van der Woude 2006; Broadbent et al. 2010).

Pap phase variation and Ag43 phase variation in *E. coli* are two most studied systems. Pap is pyelonephritis-associated pili (Pap or P pili) and plays important role in virulence in urinary track infection (Braaten et al. 1994). *ag43* gene encodes the outer membrane protein Ag43, which causes autoaggregation, enhances biofilm formation, and may affect phage adsorption (Hallet 2001; Haagmans and van der Woude 2000). Expression of the *pap* operon and *ag43* varies and is dependent on the methylation status of Dam target sequences (GATC) present in their regulatory region (Braaten et al. 1994; Haagmans and van der Woude 2000). Pap expression further requires the global regulator leucine-responsive regulatory protein (Lrp), pap specific regulatory proteins PapI and PapB and the catabolite activator protein (CAP). Methylation status of two Dam sites in the regulatory region controls the binding of the above mentioned regulatory proteins and thus, transcription (Jafri et al. 2002; Casadesu's and Low 2006). *ag43* expression is controlled by the methylation status of three Dam sites present overlapping with the binding site of oxidative stress response protein (OxyR). OxyR is a negative regulator of *ag43* and methylation of GATC sites abrogates the binding of OxyR and results in the "ON" phase. Binding of the OxyR to the regulatory region in the absence of methylation establishes the "OFF" phase (Waldron et al. 2002). Methylation dependent phase variation depends on the concentration of regulatory proteins and Dam to DNA ratio in the cell. Recently it has been shown in *S. enterica,* the product of glycosyltransferase operons (*gtr*) involved in the modifications of the O-antigen that can affect the Serotype is under the control of phase variation. This phase variation occurs by a novel epigenetic mechanism requiring OxyR in conjunction with the DNA methyltransferase Dam (Broadbent et al. 2010). Till now methylation dependent phase variation is only known for Dam, but it could potentially also be mediated by methyltransferases belonging to R-M systems.

4.4 Phase Variable DNA Methyltransferase: A New Dimension

A number of specific roles have been proposed for phase variation. It has long been proposed that phase variation is a mechanism for immune evasion by pathogenic organisms, as the majority of phase-variable genes are predicted to be involved in the biosynthesis of surface structures. Interesting exceptions are genes encoding

R-M enzymes. Phase-variable expression of R-M enzymes has been found in a variety of bacterial pathogens, including, *Mycoplasma pulmonis* (Dybvig et al. 1998), *H. pylori* (Tomb et al. 1997; Alm et al. 1999; de Vries et al. 2002), *Pasteurella haemolytica* (Ryan and Lo 1999) and *H. influenzae* (De Bolle et al. 2000), *N. gonorrhoea* (Adamczyk-Poplawska et al. 2009) , *N. meningitides* (Srikhanta et al. 2009, 2010), and *Morexella catarrahalis* (Seib et al. 2002).

Type III R-M systems represent an atypical class of phase variable genes. Type III R-M systems consist of two subunits, Mod (M), which is a functional DNA-methyltransferase, able to recognize and modify the target sequences, and Res (R), which is responsible for DNA cleavage, but functions only in a complex with the Mod subunit. Phase variable Type III R-M systems are present in a variety of pathogenic bacteria. The presence of repeats within a gene is a strong indicator that the gene is phase variable at a frequency determined by the hypermutability of the repeat regions. It has been experimentally proven in organisms, *H. influenzae* (De Bolle et al. 2000; Srikhanta et al. 2005) and *H. pylori* (de Vries et al. 2002) that the presence of repeats in R-M systems results in phase variation. Any alteration in the number of repeats can result in a frameshift mutation and thus, results in the synthesis of a truncated protein (Fig. 4.3). In turn, this results in a phase variable ON/OFF switching of Mod dependent regulons. This is believed to be an adaptive strategy used by bacterial populations facing fluctuating environmental conditions. From sequence analysis, it was predicted that Type III methyltransferases would undergo phase variation in pathogenic organisms like *Pasteurella haemolytica* (Ryan and Lo 1999), *N. meningitidis* (Fox et al. 2007; Adamczyk-Poplawska et al. 2009), *N. gonorrhoeae* (Saunders et al. 2000) and *Moraxella catarrhalis* (Seib et al. 2002). It has been shown that within a single strain of human pathogens, *N. meningitidis, N. gonorrhoeae, H. pylori* and *M. catarrhalis*, multiple phase variable Type III *mod* genes are present. Therefore, it was proposed that DNA restriction is not the only function of phase variable Type III R-M systems (Fox et al. 2007). Phasevarion is a group of genes whose expression is controlled by a phase variable DNA methyltransferase. A recent report suggests that multiple phasevarions exist within the pathogenic *Neisseria*, each regulating a different set of genes (Srikhanta et al. 2009). Both *N. meningitidis* and *N. gonorrhoeae* have two distinct *mod* genes-*modA* and *modB* and these genes switch independently. There are also distinct alleles of *modA* (major alleles include, *modA11*, *12, 13*, and minor, *modA4, 15, 18*) and *modB* (*modB1, 2*). These alleles differ only in their DNA recognition domain. *modA11* was only found in *N. meningitidis* and *modA13* only in *N. gonorrhoeae.* ModA13 recognises and methylates 5′-AGAA^{m6}A-3′. When expressed, ModA13 methylates all AGAAA sites in the genome, and thereby controls the gene expression. It was shown that two strains with the same DNA recognition domain (*modA13* allele) regulated the same set of genes, while, *N. meningitides modA11* and *modA12* were found to regulate the expression of different sets of genes, consistent with differences in their DNA recognition domain (Srikhanta et al. 2009). It has been shown that *modA13* ON and OFF strains of *N. gonorrhoeae* have distinct phenotypes in antimicrobial resistance, differential virulence in a primary human cervical epithelial cell model of infection, and in biofilm formation. A number of DNA methyltransferases

from Type II class in *H. pylori* have been predicted to exhibit phase variation (Salaun et al. 2004, 2005). Widespread distribution of phase variable R-M systems in host-adapted pathogenic bacteria suggests that this regulated random switching of multiple genes may be a commonly used strategy for generation of distinct, cell types with distinct niche specialization in host adapted bacterial pathogens. Why most of the phase variable methyltransferases found so far belongs to Type III R-M systems, is still an open question. Whether their unique genetic make up, cleavage specificities, or unusual properties of methyltransferases, dictate them to be phase variable, needs to be addressed. By modulating the activity of R-M systems by phase variation bacteria can regulate their ability to take up DNA. OFF phase R-M systems facilitate foreign DNA uptake and thus contributes to genetic variability. Alternately, the ON phase may limit the uptake of DNA from the surrounding. The balance between the two phases is critical for the survival and may be controlled by host and environmental factors. In case of Types I and III R-M systems, phase variation in Mod subunit can turn ON or OFF both the activities of the R-M system that is, methylation and DNA cleavage. On the other hand, in case of Type II R-M system phase variation in methyltransferase can create a lethal phenotype. In the absence of methyltransferase, the cognate restriction enzyme will cleave the DNA. Inactivation of modification components can cause continuous 'bacterial suicides' that may encourage intergenomic recombination and thus help in creating variability in bacteria.

4.5 Molecular Evolution of DNA Methyltransferases

DNA methyltransferases represent a highly diverse group of enzymes. Compared to methyltransferases acting on other substrates like RNA, lipid, protein and small molecules, DNA methyltransferases exhibit sequence permutation, despite similarity in structure. Amino acid sequence alignments of DNA methyltransferases have shown the presence of several conserved motifs. Motifs I-VIII and X are present in most subfamilies with a region of high variability (Malone et al. 1995). The variable region is implicated in recognition of the target sequence and termed as Target recognition domain (TRD). Motifs IV-VIII were involved in catalysis and motifs X and I-III form AdoMet-binding pocket (Schluckebier et al. 1995). Based on the sequential order of the cofactor-binding domain, the catalytic domain, and the recognition domain (TRD), six groups of DNA methyltransferases have been proposed (Fig. 4.1) (Bujnicki 2002). Two models have been proposed to explain the sequence permutation which has resulted in evolution of DNA methyltransferase (Bujnicki and Radlinska 1999; Jeltsch 1999).

Jeltsch proposed that circular permutations play a critical role in the evolution of new methyltransferases (Jeltsch 1999). This model based on the concept that a permutated protein can arise naturally from tandem repeats. This model includes the duplication and in frame fusion of a methyltransferase gene, resulting in a fusion methyltransferase with two catalytic domains and TRDs. Introduction of a start codon in first copy and stop codon in next can result in a circularly permutated variant of a methyltransferase. It was proposed that β sub group of methyltransferase have been

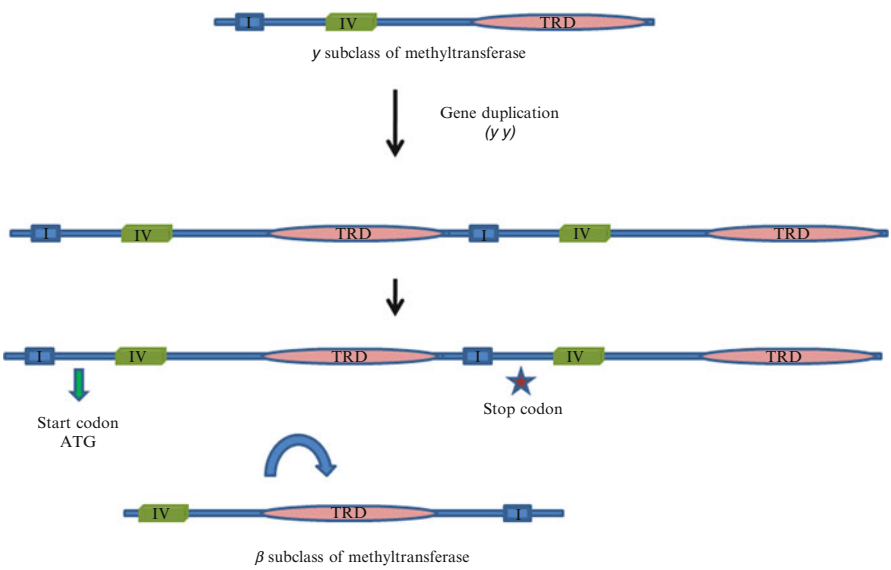

Fig. 4.4 Evolution of β subclass of methyltransferase from γ subclass of methyltransferase by circular permutation

evolved from the duplication of γ subgroup of methyltransferase or vice versa (Fig. 4.4) (Jeltsch 1999). M.FokI represents an interesting example which can be considered as the closest intermediate in circular permutation model. M.FokI contains two complete sets of motifs present in an adenine methyltransferase (Kita et al. 1989; Sugisaki et al. 1989). Recently it has been shown that in *H. pylori*, a single nucleotide insertion between two adjacent methyltransferases can result in a fused methyltransferase with two sets of catalytic motifs, AdoMet binding motifs and TRDs (Kumar and Rao 2011). But this model fails to explain the differences between the TRDs of β and γ sub group of methyltransferase. It is, therefore, unlikely that simple circular permutations from γγ to β or from γγ to β occurred in nature (Bujnicki 2002).

A second model suggests that DNA methyltransferases have evolved by intra-or intergenic rearrangements of gene fragments (Lupas et al. 2001). This model proposes the fragmentation of DNA methyltransferase gene by the action of nuclease and then reassembly of the fragments into a functional form. Reassembly results in shuffling of the motifs. Bujnicki (2002), proposed that M.*MwoI* (δ) has evolved from M.*SfiI* (β) by shuffling of gene fragments and M.*TvoORF1413P* (ζ) evolved from M. *ThaI* (β) sub group of methyltransferase by gene duplication or cut and paste mechanism. It is believed that the two mechanisms are not mutually exclusive and could be possible that both played significant roles in the evolution of permuted methyltransferase.

One of the most intriguing questions is the evolution of R-M enzymes, where the restriction endonuclease and the cognate methylase usually recognize the same DNA sequence. It is straightforward to expect that such enzymes would share at least some elements of the target recognition machinery. Previous attempts to find

evidence for such inheritance by pair wise comparison of corresponding restriction enzymes and methyltransferases were unsuccessful. Moreover, common themes of sequence recognition so far have not been found among restriction endonuclease themselves (Wilson and Murray 1991). One possibility is that each component evolved independently from the other and later combined to form R-M systems. Alternatively, due to strong divergence the original domains are barely detectable at the primary structure level.

4.6 Conclusions and Perspectives

The ability to covalently add methyl groups to targeted adenine or cytosine residues in specific DNA sequences without any other changes in the primary DNA structure is a remarkable feature of DNA methyltransferases. Recent evidences suggest that, in addition to protecting the bacterial genome from external DNA, R-M systems have other biological functions (Ando et al. 2010; Jeltsch 2003). The genome analysis of a number of sequenced strains of *H. pylori* showed that this bacterium possesses an unusually high number of strain-specific R-M genes. *H. pylori* is well adapted to the gastric environment, and acquisition of numerous R-M systems might be related to its unique adaptability to acidic environment. Phase variable Type III R-M systems are present in a variety of pathogenic bacteria. Widespread distribution of phase variable R-M systems in host-adapted pathogenic bacteria suggests that this regulated random switching of multiple genes may be a commonly used strategy for bacterial pathogens. Identification of phase variable R-M systems and their role in gene regulation has added another dimension in the field of epigenetic regulation in bacteria. The identification and study of both species-specific and strain-specific phase variable methyltransferases of pathogenic bacteria may therefore, improve the understanding of their pathogenic mechanisms.

The finding that N^6 methyladenine is essential for the virulence and pathogenesis in many bacteria raises the possibility of using an inhibitor of adenine methyltransferase as antimicrobial agents. Decrease in the virulence by the deletion of *dam* in a number of pathogenic bacteria, combined with their capacity to persist at low levels in animals organs make them an interesting target to use as Dam-based live vaccines.

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Part II
Developmental Epigenetics

Chapter 5
Metabolic Aspects of Epigenome: Coupling of *S*-Adenosylmethionine Synthesis and Gene Regulation on Chromatin by SAMIT Module

Kazuhiko Igarashi and Yasutake Katoh

Abstract Histone and DNA methyltransferases utilize *S*-adenosyl-*L*-methionine (SAM), a key intermediate of sulfur amino acid metabolism, as a donor of methyl group. SAM is biosynthesized by methionine adenosyltransferase (MAT) using two substrates, methionine and ATP. Three distinct forms of MAT (MATI, MATII and MATIII), encoded by two distinct genes (*MAT1A* and *MAT2A*), have been identified in mammals. MATII consists of α2 catalytic subunit encoded by *MAT2A* and β regulatory subunit encoded by *MAT2B,* but the physiological function of the β subunit is not clear. MafK is a member of Maf oncoproteins and functions as both transcription activator and repressor by forming diverse heterodimers to bind to DNA elements termed Maf recognition elements. Proteomics analysis of MafK-interactome revealed its interaction with both MATIIα and MATIIβ. They are recruited specifically to MafK target genes and are required for their repression by MafK and its partner Bach1. Because the catalytic activity of MATIIα is required for the MafK target gene repression, MATIIα is suggested to provide SAM locally on chromatin where it is recruited. One of the unexpected features of MATII is that MATIIα interacts with many chromatin-related proteins of diverse functions such as histone modification, chromatin remodeling, transcription regulation, and nucleo-cytoplasmic transport. MATIIα appears to generate multiple, heterogenous regulatory complexes where it provides SAM. Considering their function, the heterooligomer of MATIIα and β is named SAMIT (SAM-integrating transcription) module within their interactome where it serves SAM for nuclear methyltransferases.

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5.1 Introduction: Overlooked Metabolites in Epigenetics

Post-translational modifications of histone tails especially acetylation and methylation regulate gene expression as a part of the epigenome system. Histone acetyltransferases utilize acetyl-Coenzyme A (Ac-CoA), a molecular hub of carbohydrate, lipid, and energy metabolism. Histone methyltransferases utilize *S*-adenosyl-*L*-methionine (SAM or AdoMet), a key intermediate of sulfur amino acid metabolism. DNA methyltransferases also utilize SAM. Viewing these simple facts, it seems reasonable to speculate that cellular metabolism and chromatin regulation are intimately connected. However, a link between chromatin and metabolism has been overlooked. Such a link would be layered at several mechanistic aspects. First, reactions of methylation and acetylation within nuclei may be substrate-limited. Amounts of Ac-CoA or SAM within a cell may fluctuate depending on nutrition and/or energy states, limiting or enhancing histone acetylation and methylation. Consistent with the idea, histone acetylation increases when cells, cultured with low glucose, are supplied with acetate. The acetate can be metabolized to Ac-CoA in the cell (Wellen et al. 2009), indicating that Ac-CoA is limiting for histone acetylation under certain conditions. Therefore, epigenetic modifications may respond to nutritional conditions and metabolic status. Second, enzymes for the synthesis of Ac-CoA and SAM may be compartmentalized within the nuclei. Subcellular localization of Ac-CoA or SAM synthesizing enzymes may affect gene expression. As for example, Ac-CoA synthase 2p in yeast is localized in nuclei (Takahashi et al. 2006), suggesting that local, nuclear synthesis of Ac-CoA may facilitate histone acetylation. In other words, availability and activity of metabolic enzymes related to Ac-CoA or SAM within nuclei may significantly affect histone modification and hence gene expression. Third, these enzymes may be parts of protein complexes that would allow an efficient flow of substrates (i.e., substrate channeling). In this case, assembly and disassembly of such complexes would confer dynamic regulation of epigenome. Despite these interesting possibilities pertaining to the epigenome, little is known about whether SAM is limiting in the nuclei or whether enzymes that synthesize SAM play a role in the nucleus.

Methylation of both histone and DNA is more dynamic than originally thought, as active demethylation of histone and DNA has been discovered (Lan et al. 2008). This raises the possibility that a subtle change in the process of methylation can affect local and global levels of methylation of histone and DNA. In this review, we discuss a connection between the SAM synthesis pathway and histone methylation that has been highlighted by a recent identification of the SAMIT (SAM-integrating transcription) module for chromatin regulation (Katoh et al. 2011).

5.2 S-Adenosylmethionine (SAM) in Epigenetics

5.2.1 SAM as a Hub of Metabolism

SAM was discovered in 1953 (Cantoni 1953), the year marked by the discovery of DNA double helix. SAM is the second most widely used enzyme substrate after ATP (Cantoni 1975). SAM is the major methyl donor among other donors such as folate in enzymatic reactions of methyl transfer. It is involved in the biosynthesis of diverse bioactive substances including hormones and neurotransmitters. SAM is utilized as a substrate by all of the known methyltransferases of histones and DNA. The use of SAM as a methyl donor is because of the strong electrophilic character of its methyl group. This poses a risk: cells need to avoid non-specific reactions between SAM and nucleophiles such as DNA. In vitro SAM acts as an alkylating mutagen (Rydberg and Lindahl 1982). However, its mutagenic activity in *E. coli* cell has been challenged (Posnick and Samson 1999). As the mutagenic potential of SAM in eukaryotes awaits further study, it leaves the pathological significance of SAM as a mutagen unclear. It is therefore reasonable to consider that the evolution of life has selected systems that balance between essential functions and detrimental effects of SAM in the nuclei.

SAM is biosynthesized by methionine adenosyltransferase (MAT) using two substrates, methionine and ATP (Fig. 5.1). Upon transmethylation reaction by

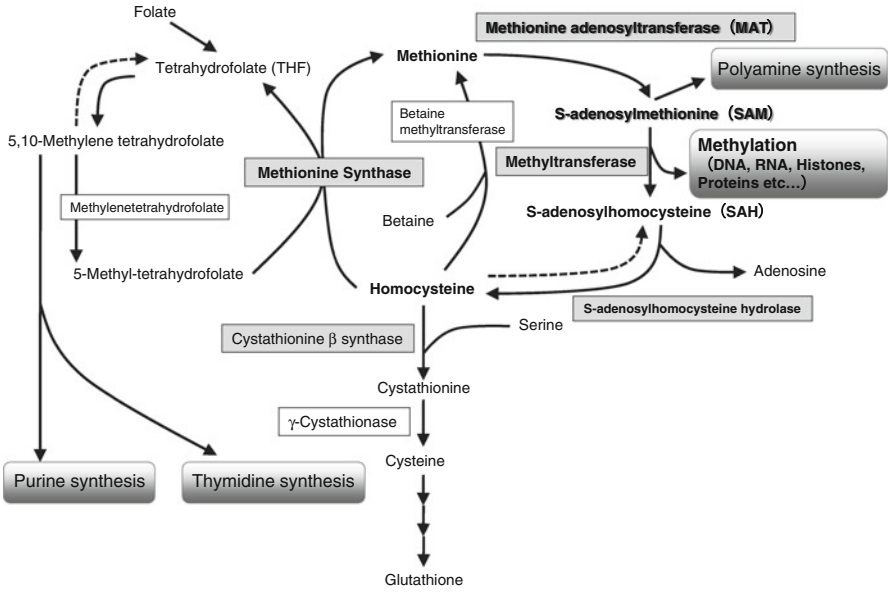

Fig. 5.1 S-adenosylmethionine (SAM) cycle and metabolism. Metabolites and their connections are shown with respective enzymes (*boxed*). Reverse reactions are shown with *dashed lines*

methyltransferases, SAM is converted into *S*-adenosylhomocysteine (SAH). SAH is then cleaved by SAH hydrolase, generating homosysteine and adenosine. Homocysteine is methylated to regenerate methionine by methionine synthase using the methyl group of 5-methyltetrahydrofolate, connecting the overall reactions into a single methionine cycle. Completion of the reaction cycle occurs only in the liver, since most other tissues lack methionine synthase and/or other enzymes in the cycle (Mato et al. 1997). The metabolites of the methionine cycle are also connected to important pathways other than transmethylation. SAM is the precursor of spermidine and spermine synthesis. SAH provides adenosine for the synthesis of ATP. Homocysteine is metabolized through the transsulfuration pathway to generate cysteine, which is then utilized for synthesis of diverse molecules including protein, glutathione, and coenzyme A. Methyltetrahydrofolate and tetrahydrofolate, a product of methionine regeneration, are parts of the folate cycle which participates in the synthesis of thymidylate (dTMP) and purines.

5.2.2 Enzymes for SAM Synthesis

Three distinct forms of MAT (MATI, MATII and MATIII), encoded by two distinct genes (*MAT1A* and *MAT2A*), have been identified in mammals (Mato et al. 1997; Suma et al. 1983; Mitsui et al. 1988; Sakata et al. 1993; Kotb et al. 1997; Markham and Pajares 2009). MATI and MATIII are a tetramer and a dimer respectively of α1 catalytic subunit, which is encoded by *MAT1A*. MATII consists of the catalytic subunit α2, encoded by *MAT2A* and the regulatory subunit β, encoded by *MAT2B*. The subunit structure is presumably $\alpha 2_2\beta_1$. The MATII β subunit increases MATII catalytic activity by lowering *K*m for L-methionine (LeGros et al. 2000), but sensitizes it to SAM-mediated product inhibition by lowering *K*i for SAM (Halim et al. 1999). Physiological function of the β subunit in MATII is not clear (see below). MATI has been considered to be liver-specific, whereas MATII is a ubiquitous enzyme. However, it has been reported recently that MATI is expressed in a wider range of tissues in rat including lung and pancreas (Reytor et al. 2009). The mammalian MAT isozymes differ in their *K*m for methionine, which is ~30 μM for MATII, ~100 μM for MATI, and 1 mM for MATIII (Markham and Pajares 2009). While the physiological significance of the differences in *K*m values for methionine is not clear at present, they may reflect different microenvironments (i.e., methionine concentrations) where the respective isozymes function. MATII may be suitable for reactions where methionine is relatively sparse. Even yeast cells possess two isozymes of MAT (Chiang and Cantoni 1977), suggesting that their division of labor is fundamental to life.

Several investigators reported intracellular concentrations of SAM. SAM levels in lymphoid cells increase upon activation: it is around 5 μM and 30 μM in resting and activated lymphoid cells, respectively (German et al. 1983). Much higher levels of around 300 μM are reported in human acute myeloid leukemia cell line HL-60 (Chiba et al. 1988). Although these observations may suggest that SAM is not limiting

within a cell, little is known about nuclear SAM levels. Subcellular distribution of SAM has been addressed recently (Brown et al. 2010). In their report, the authors measured SAM levels before and after treatment of mice with acetaminophen, a leading cause of drug-induced liver disease and known to reduce SAM levels. Despite of being out of their scope, they report that liver tissue contains roughly 60 nmol of SAM per g tissue, which partition into fractions of soluble cytoplasm, mitochondoria, and nuclei at roughly 20, 10, and 0.2 per g tissue, respectively. Thus, the nuclear compartment contains significantly less SAM compared to other compartments. It remains possible however, that SAM may leak out of nuclei during the biochemical fractionation. It is a fundamental question whether SAM constitutes a single pool in cytoplasmic and nuclear compartments. In an extreme and simplistic view, SAM may be synthesized by cytoplasmic MAT enzyme and can be provided to nuclear methyltransferases by diffusion. Such a system is simple but appears to lack regulatory capability. It may be difficult to balance SAM requirement in nuclei when it is synthesized elsewhere.

5.3 SAM Synthesis in Nuclear Compartment

5.3.1 MafK-MATII Interaction and SAMIT Module

MafK is a member of Maf oncoproteins that functions as both transcription activator and repressor. It forms diverse heterodimers that bind to DNA elements termed Maf recognition elements (MAREs) (Fujiwara et al. 1993; Igarashi et al. 1994; Igarashi and Sun 2006; Motohashi and Yamamoto 2007). Many of the MafK target genes such as heme oxygenase-1 (HO-1) and ferritin are oxidative stress-responsive genes. These target genes are repressed and activated by MafK-Bach1 and MafK-Nrf2 heterodimers, respectively (Igarashi and Sun 2006; Motohashi and Yamamoto 2007). Proteomics analysis of MafK-interactome revealed its interaction with both MATIIα and MATIIβ (Katoh et al. 2011). They are recruited specifically to MafK target genes such as HO-1 and globin genes (Fig. 5.2a). They are required for HO-1 gene repression by MafK and Bach1. Because the catalytic activity of MATIIα is required for the MafK target gene repression (Katoh et al. 2011), MATIIα is suggested to provide SAM locally on chromatin where it is recruited. Interestingly, chromatin of the enhancer regions of HO-1 gene contain repressive histone methylation such as histone H3 lysine 9 (H3 K9) and H3 K4 dimethylation which play roles in gene repression (Kim and Buratowski 2009). While exact connection between this particular methylation and MATIIα is not clear, the latter may be involved in writing of the repressive methylation when recruited locally.

One of the unexpected features of MATII is that MATIIα interacts with many chromatin-related proteins of diverse functions such as histone modification, chromatin remodeling, transcription regulation, and nucleo-cytoplasmic transport (Katoh et al. 2011) (Table 5.1). *Drosophila melanogaster* MATIIα (see below) binds to multiple proteins with known and unknown functions in yeast two hybrid assays

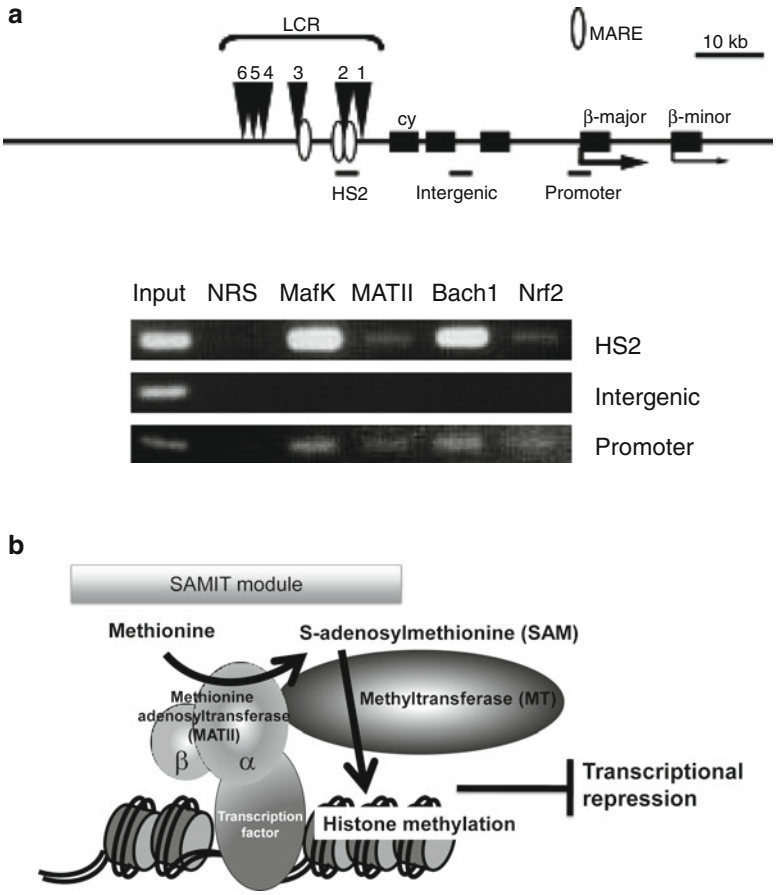

Fig. 5.2 Coupling of SAM synthesis and methylation by SAMIT module. (**a**) MATII was recruited to β-globin locus. Schematic representation of mouse β-globin locus. PCR primer pairs for ChIP analyses were designed to amplify the LCR hypersensitive sites, intergenic region, and β-major globin promoter. ChIP assays were performed by using anti-MATII, anti-MafK, anti-Bach1, and anti-Nrf2 antibodies and control rabbit IgG (NRS) with MEL cells. Gel images of PCR products of HS2, intergenic and promoter using input and precipitated chromatin as template are shown. NRS, normal rabbit serum. (**b**) The core of the SAM-dependent regulatory complex, α and β subunits, are named SAMIT module. This module is integrated into a complex with histone H1 and H3 methyltransferase activities. This complex catalyzes both SAM synthesis and histone methylation in vitro. SAMIT is likely to couple these two steps of the reaction by physically interacting with histone methyltransferases

(BioGRID:71491), suggesting its direct interaction with diverse proteins. Because MATIIα sediments in glycerol gradient analysis as several peaks including high molecular weight species, it appears to participate in multiple distinct complexes (Katoh et al. 2011). These observations suggest that the MATIIα interactome is heterogenous and modular in terms of function and structure.

Table 5. 1 Shared interactome of MafK and MATIIα

Functional/ proteomic cluster	Protein	Feature
(***Complex***)		
Swi/Snf	Baf53a	Requirement for maximal ATPase activity of BRG1
	Baf57	Remodeling histone H1-containing chromatin at the CD4 silencer
	Baf60b	Bridging interactions between transcription factors and SWI/SNF complexes
	Baf155	p53 target and a tumor suppressor by modulating p21(WAF1/CIP1) expression
	Baf180	Coronary vessel formation
NuRD	CHD4	Regulation of the DNA-damage response (DDR) and G1/S cell-cycle transition
	MBD3	A component of the NuRD co-repressor complex for development of pluripotent cells
	Gatad2b	Mediation of MBD2 and histone interaction
CHRAC	ACF1	Requirement for DNA replication through heterochromatin
PARP	PARP1	The attachment of ADP ribose units to target proteins
	Ku70	One of two subunits of Ku. Ku plays a critical role in the regulation of many cellular processes
	Ku80	Another of two subunits of Ku
	Ssrp1	The smaller of the two subunits of FACT. FACT mediates nucleosome reorganization
	Supt16h	The larger of the two subunits of FACT
(***Specific domain***)		
Chromodomain	CHD5	A tumor suppressor at human 1p36
	CHD6	A DNA-dependent ATPase and localization at nuclear sites of mRNA synthesis
	CHD7	A critical regulator of important developmental processes in organs affected by human CHARGE syndrome
	CHD8	Regulation of a serum response factor activity and smooth muscle cell apoptosis
	CHD9	A recently identified chromatin remodeler in osteogenic cell differentiation
(***Function***)		
DNA repair	Rad50	A regulator of cell cycle checkpoints and DNA repair
	Fen1	A member of the Rad2 structure-specific nuclease family, possesses 5'-exonuclease and gap-endonuclease activities
	HSP70	Ubiquitous molecular chaperones
Chromosome	p400	Modulation of cell fate decisions by the regulation of ROS homeostasis
	TopoIIα	The separation of chromosomes for DNA replication
	H3.3B	A replacement histone subtype
Transcription	Bach1	Transcriptional repression of oxidative-stress-response genes

(continued)

Table 5. 1 (continued)

Functional/ proteomic cluster	Protein	Feature
Histone acetylation	Myst2	H4-specific histone acetylase, and a coactivator of the DNA replication licensing factor Cdt1
Histone methylation	G9a	Histone methyltransferase for dimethylation of histone H3 K9
	Ehmt1/GLP	Histone methyltransferase for dimethylation of histone H3 K9
	ALL1	Histone methyltransferase for dimethylation of histone H3 K4
	MATIIβ	A regulatory subunit of MATII
Proteolysis	Senp1	Redox sensors and effectors modulating the desumoylation pathway and specific cellular responses to oxidative stress
Transcriptional cofactor	Sin3a	A transcriptional regulatory protein
	PML	A member of the tripartite motif (TRIM) family
	DMAP1	A co-repressor that stimulates DNA methylation globally and locally at sites of double strand break repair
Transport	RanBP2	Direct interaction with the E2 enzyme UBC9 and strongly enhancing SUMO1 transfer
Spindle microtubule	Kif4	Regulation of activity-dependent neuronal survival by suppressing PARP-1 enzymatic activity
Other	Ubqln4	Proteasome-mediated degradation of proteins and interaction with ataxin-1

In the context of transcription regulation by MafK and Bach1, several MATIIα-interactants such as MATIIβ, poly-ADP-ribose polymerase 1 (PARP1), BAF53a, and CHD4 (Katoh et al. 2011) are critical for transcription repression. They form higher molecular weight complexes of roughly 600 kDa, among which at least MATIIβ, BAF53a, and CHD4 are recruited to HO-1. While MATIIα and β participate in MafK-mediated gene repression, the β subunit also plays a role independent of α subunit (see below). Therefore, the core of the SAM-dependent regulatory complex, comprising the α and β subunits, are named SAMIT module for SAM-integrating transcription repression. Because CHD4 is a rather large protein with many functional domains such as ATPase, chromo domain, and PHD finger, it may function as a platform to assemble SAMIT module into a larger operative complex and to organize its function with other proteins including PARP1 and BAF53a.

The fact that MATIIβ is recruited to MafK target genes strongly suggests that it is not a classical regulatory subunit for fine-tuning of its catalytic subunit, as has been considered previously (LeGros et al. 2000; Halim et al. 1999). A structural feature of MATIIβ also supports this idea. Human MATIIβ show 28% homology with a family of bacterial enzymes that catalyze the reduction of TDP-linked sugars such as dTDP-4-dehydrorhamnose reductase and other proteins involved in the production of polysaccharides. Therefore, MATIIβ itself may be an enzyme in

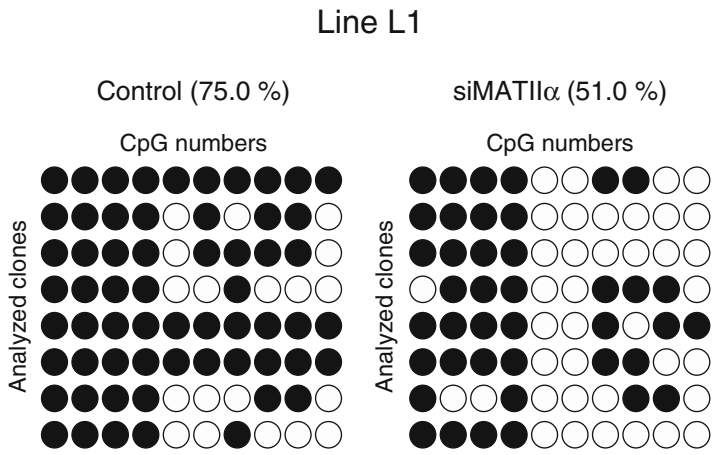

Fig. 5.3 Involvement of MATIIα in DNA methylation. Bisulphite sequencing analysis of LINE L1 locus in Hepa1 cells transfected with control or MATIIα siRNAs. The overall percentages of methylated CpGs (*black circle*) are indicated in parentheses

carbohydrate metabolism. MATIIβ interacts with RNA binding protein HuR. MATIIβ promotes cytoplasmic accumulation of HuR and translation of HuR target mRNAs such as cyclin A (Xia et al. 2010). Furthermore, MATIIβ interacts with diverse nuclear proteins (our unpublished observation) and re-evaluation of its function beyond the regulatory subunit of MATII is necessary.

5.3.2 Coupling of SAM Synthesis and Methylation by SAMIT

SAMIT module is integrated into a complex with histone H1 and H3 methyltransferase activities (Katoh et al. 2011). Furthermore, this complex catalyzes both SAM synthesis and histone methylation in vitro. In the newly developed assay, radiolabeled methionine is used instead of radio-labeled SAM. Methylation of target proteins such as histones can be detected only when a protein complex contains both SAM synthesizing and methyltransferase activities. SAMIT and its interacting proteins mediate efficient methylation of histone H1 and H3 in vitro (Katoh et al. 2011). Thus, SAMIT is likely to couple the two steps of the reaction by physically interacting with histone methyltransferases (Fig. 5.2b). Such a coupling may confer several advantages. First, juxtaposition of SAM synthesis to methylation can avoid genotoxic effects of SAM by lowering overall SAM levels but allowing local methylation reaction. Second, association and dissociation of SAMIT with methyltransferases provide a window for dynamic regulation. Methylation of chromatin may be promoted or inhibited depending on their interaction, conferring signal responsiveness of histone and/or DNA methylation.

Another interesting possibility is in the regulation of DNA methylation. MATIIα is also involved in DNA methylation. Upon its knockdown, DNA methylation at LINE L1 repeats is reduced (Fig. 5.3). Therefore, it will be important to examine

interaction of SAMIT module with DNA methyltransferases. Active DNA demethylation has been suggested as a mechanism for conversion of methyl-cytosine to cytosine. Among several candidate enzymes for this reaction, DNA methyltransferase 3A (DNMT3A) is unique, in that it catalyzes both methylation and demethylation. However, demethylation can occur only when SAM concentration is very low. In this sense, SAM levels must fluctuate in a wide range. Considering that SAM is essential for diverse biochemical reactions, it is unlikely that gross SAM levels within cells become very low to allow DNA demethylation by DNMT3A (Wu and Zhang 2010). Coupling of MATII with methyltransferases is expected to resolve this conundrum since such a system allows lowering local SAM levels by dissociating MATII from target genes and/or methyltransferases.

5.3.3 *Genetics of MATII in Drosophila melanogaster*

In *Drosophila melanogaster*, modifier mutations of position effect variegation and *Polycomb* group (Pc-G) genes have been useful to identify regulators of chromatin structure. MATII was identified in such an analysis. *zeste* encodes a DNA binding protein that recognizes polycomb responsive elements (PREs) and regulatory regions of *white* gene. The neomorphic $zeste^{1}$ mutation renders the encoded protein extremely sticky and abnormally efficient in white gene repression. *Su(z)5* was reported as a suppressor mutation of $zeste^{1}$ and a homozygous embryonic lethal mutation (Person 1976), suggesting its involvement in transcription repression. This mutation was followed up further by others, revealing that *Su(z)5* is an enhancer of a polycomb mutation (Larsson et al. 1996), again suggesting its involvement in gene repression. The cloned wild-type allele of *Su(z)5* was found to encode MAT. Fly has two MAT isozyme genes and according to genome data base (Gene ID: 48552), *Su(z)5* encodes MATIIα. Even though *Su(z)5* enhance the *Pc* mutant, *Su(z)5* does not induce ectopic expression of the homeotic genes by itself (Larsson and Rasmuson-Lestander 1998). This finding may suggest that the MATIIα function is at least partially compensated by MATI or other chromatin proteins. The authors suggested that the phenotypes of *Su(z)5* mutation was due to an obstruction of the polyamine biosynthesis (Larsson et al. 1996). However, considering the findings regarding mouse MATIIα, it is more likely due to a collapse in methylation of chromatin.

5.3.4 *Nuclear MATI*

MATI is mainly cytoplasmic but a portion of it is localized within nuclei (Reytor et al. 2009). It is becoming clear that MATI also functions in the nucleus and regulates gene expression. Overexpression of MATI in CHO cells causes a mild but selective increase in trimethylation of H3 K27, suggesting that MATI is functionally coupled

with a specific methyltransferase for this modification. This observation also suggests that SAM is limiting for histone methylation in nuclei. The presence of nuclear localization signal on MATI (Reytor et al. 2009) is consistent with its nuclear function. However, detailed nuclear function and mechanism of MATI are unclear.

5.4 Metabolic View of Epigenome

5.4.1 Nuclear Methionine Cycle?

All of the metabolic substrates involved in the methionine cycle are small and are expected to diffuse freely in a cell. From the viewpoint of biochemistry, however, these enzymatic reactions may be coupled for efficiency and regulation by enzyme compartmentalization, just as the case for the citric acid cycle. Indeed, SAH hydrolase is localized with in the nuclei in Xenopus laevis (Radomski et al. 1999). Because most methyltransferases bind SAH with higher affinity than SAM, they are subject to potent product inhibition (Chiang et al. 1996). Therefore, a nuclear localization of SAH hydrolase may facilitate rapid turnover of SAH, promoting histone methylation by histone and DNA methyltransferases within nuclei. However, it is not known whether nuclear SAH hydrolase is universal among other types of cells or it plays any physiological role within nucleus. Myc induces the expression of SAH hydrolase to promote mRNA Cap methylation (Fernandez-Sanchez et al. 2009), raising the possibility that the induced enzyme functions in nuclei. It will be an important question whether these enzymes are coupled on chromatin to regulate histone and DNA methylation.

5.4.2 Versions on a Theme: Ac-CoA and Deoxyribonucleotides in Nuclei

In the yeast *Saccharomyces cerevisiae*, Ac-CoA synthetase (Acs) catalyzes synthesis of Ac-CoA from acetate. One of its isozyme Acs2p is required for histone acetylation and global gene expression (Takahashi et al. 2006). While Acs2p is normally present in the nuclear compartment, this localization appears irrelevant for its function. A temperature-sensitive mutant of *acs2* can be rescued by prokaryotic Acs tagged with a nuclear export signal (Takahashi et al. 2006). This observation suggests that the nuclear and cytosolic Ac-CoA exists in a single pool. However, this observation should be carefully interpreted because the engineered protein may transit nuclei.

In contrast to yeast, most mammalian cells do not utilize acetate as a source of Ac-CoA synthesis. Ac-CoA outside of mitochondria is synthesized from mitochondoria-derived citrate by the enzyme ATP-citrate lyase (ACL). ACL is present in nuclei and required for histone acetylation (Wellen et al. 2009). However,

it has not been tested whether nuclear localization of ACL is critical for its function. This is important to understand whether Ac-CoA is present in a single pool in the nuclear and cytosolic compartments (Takahashi et al. 2006).

In DNA repair and replication, a balanced supply of deoxyribonucleotide is essential. It has been reported that ribonucleotide reductase subunits accumulate very rapidly at a DNA damage site, suggesting that efficient DNA repair is dependent upon localized supply of substrates for DNA synthesis (Niida et al. 2010). This recruitment of ribonucleotide reductase subunits is dependent on their binding to histone acetylase Tip60. Furthermore, binding of these subunits to Tip60 is necessary for efficient DNA repair (Niida et al. 2010). However, not all enzymes for nucleotide synthesis are compartmentalized within nuclei. Purines are essential building blocks for RNA and DNA. De novo synthesis of adenosine and guanosine involves ten chemical reactions that transform phosphoribosyl pyrophosphate to inosine monophosphate. Enzymes involved in these reactions are mainly cytoplasmic and interact with each other when purines are low to form clusters or "purisome" to carry out de novo purine biosynthesis (An et al. 2008). Although the place for the reactions is cytoplasmic, this example reiterates the importance of interactions of enzymes in a pathway for efficient reactions and regulation. Therefore, even when cytoplasmic and nuclear compartments share a common pool of metabolites, localized assembly of enzymes is expected to promote efficient, tunable flow of metabolites and their ultimate incorporation into macromolecules as constituents or modifications.

5.5 Perspectives

While nuclear functions of the enzymes involved in the synthesis of SAM and Ac-CoA are emerging, our understanding is still fragmentary. We do not know much about concentrations of these metabolites in nuclei and how they change during processes such as stress response and differentiation. Because small metabolites would leak out of nuclei during biochemical fractionation, probes for in situ imaging of the relevant metabolites will be required to address this issue.

While MATII interacts with diverse nuclear proteins involved in different processes such as DNA replication and repair (Katoh et al. 2011), nothing is known about physiological and pathological significance of these interactions. It is very likely that localized SAM synthesis is coupled with writing of methyl mark on chromatin during these reactions.

Based on the fact that leukemic cells utilize significantly higher levels of SAM than normal lymphocytes, it has been shown that leukemic cells are more sensitive to reduction of MATIIβ by RNA interference (Attia et al. 2008). Therefore, therapeutic exploitation of the nuclear metabolic enzymes will be an interesting future issue.

In a broader perspective, mapping nuclear protein networks will be critical to understand a cross-talk of metabolism and epigenome. With procedures that allow recovery of weak and transient interaction, in combination with sensitive and accurate mass spectrometry analysis, we will uncover more and more subtle but salient

interactions. Such interactions are usually visualized as substoichiometric bands in protein gels and have been discarded because they do not conform to the criteria of "complex". However, nuclear "complexome" appears to be modular in nature and stable hetero-oligomers of proteins (modules) participate in multiple different complexes (Malovannaya et al. 2011). Substoichiometric proteins may reflect protein modules that interact with a sub-fraction of complexes to fulfill specific and/or dynamic function. Therefore, SAMIT module will be a unique bridgehead to initiate our exploration into the modularity of nuclear protein and metabolic networks.

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Chapter 6
Epigenetic Regulation of Male Germ Cell Differentiation

Oliver Meikar, Matteo Da Ros, and Noora Kotaja

Abstract Male germ cell differentiation is a complex developmental program that produces highly specialized mature spermatozoa capable of independent movement and fertilization of an egg. Germ cells are unique in their capability to generate new organisms, and extra caution has to be taken to secure the correct inheritance of genetic and epigenetic information. Male germ cells are epigenetically distinct from somatic cells and they undergo several important epigenetic transitions. In primordial germ cells (PGCs), epigenome is reprogrammed by genome-wide resetting of epigenetic marks, including the sex-specific imprinting of certain genes. Postnatal spermatogenesis is characterized by drastic chromatin rearrangements during meiotic recombination, sex chromosome silencing, and compaction of sperm nuclei, which is accomplished by replacing near to all histones by sperm-specific protamines. Small RNAs, including microRNAs (miRNAs), endogenous small interfering RNAs (endo-siRNAs) and PIWI-interacting RNAs (piRNAs) are also involved in the control of male gamete production. The activities of small RNAs in male germ cells are diverse, and include miRNA- and endo-siRNA-mediated posttranscriptional mRNA regulation and piRNA-driven transposon silencing and the control of DNA methylation in PGCs. In this chapter, we give a brief review on the epigenetic processes that govern chromatin organization and germline-specific gene expression in differentiating male germ cells.

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6.1 Introduction to Spermatogenesis

6.1.1 From Primordial Germ Cells to Spermatozoa

In mammalian embryos, germ cells are specified shortly after implantation, emerging from the epiblast that consists of pluripotent cells. Before gastrulation, precursor cells of primordial germ cells (PGCs) are induced within the proximal rim of the epiblast by morphogenetic signals provided from adjacent extra-embryonic ectoderm. During gastrulation, the nascent PGCs start to migrate toward the future gonads and rapidly proliferate (Matsui 2010). After migrating to the gonadal ridge, PGCs become gonocytes within cords that are formed by Sertoli precursors and surrounded by peritubular cells. Gonocytes show a burst of mitotic activity, then arrest in the G_0 phase of cell cycle remaining mitotically quiescent until after birth, when they give rise to spermatogonia (de Rooij and Russell 2000). The postnatal production of mature spermatozoa from spermatogonial stem cells is a complex process that takes place in seminiferous tubules inside the testis (Fig. 6.1). Sertoli cells are the somatic cells in the seminiferous epithelium that control the correct progression of spermatogenesis through cell-cell interactions and communication with the differentiating germ cells. Sertoli cell function itself is regulated by the hypothalamic-pituitary-gonadal axis, and depends on the actions of follicle stimulating hormone (FSH) and testosterone (T, produced by Leydig cells in the testis after stimulation by luteinizing hormone, LH) (Fig. 6.1).

Spermatogenesis includes proliferation, differentiation and morphogenesis of male germ cells (Hess and de Franca 2008). The process begins when diploid spermatogonia multiply by consecutive mitotic divisions and then enter the meiotic program and become spermatocytes. During meiosis, aligned homologous chromosomes pair and the synaptonemal complex is formed. Synapsis permits genetic crossover at sites along the synaptonemal complex known as recombination nodules. Finally the synaptonemal complex disintegrates, bivalent chromosomes align on the metaphase plate and sister chromatids dissociate into two daughter cells. The second meiotic division of secondary spermatocytes results in the production of haploid spermatids. The postmeiotic developmental phase, spermiogenesis, involves the differentiation of spermatids into spermatozoa. This phase includes an enormous morphogenetic transformation involving DNA compaction, cytoplasmic ejection and acrosome and flagellar formation (Kimmins et al. 2004).

Sertoli cells are attached to the basal lamina of the seminiferous tubules and extend the cytoplasm towards the lumen and interact with all the differentiating germ cell types. Spermatogonia are also localized at the level of the basal lamina, while spermatocytes, round spermatids, elongating spermatids and mature spermatozoa are present at successive steps moving from the basal lamina to the lumen. Spermatogenesis is completed when the mature spermatozoa are released from the Sertoli cells and transported to the epididymis. The whole process occurs in an ordered manner, referred to as the spermatogenic cycle, which is divided in a species-specific number of precisely timed stages (i.e. 12 in the mouse), easily

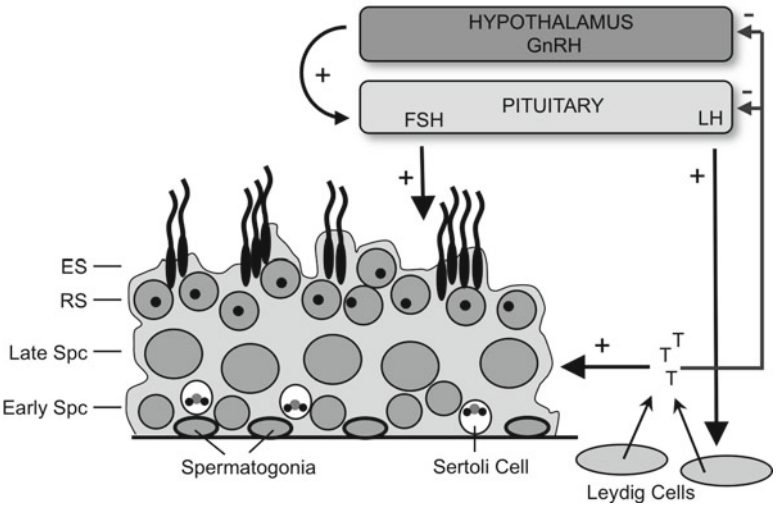

Fig. 6.1 Organization of seminiferous epithelium and hormonal control of spermatogenesis. Inside the seminiferous tubules, germ cells are embedded in the cytoplasmic pockets of somatic Sertoli cells that regulate their functions through direct cell contacts and paracrine signaling. The least differentiated germ cells, spermatogonia, are localized in the basal compartment of the tubule close to the basal lamina. In the course of differentiation, germ cells move towards the luminal compartment so that the next layer from spermatogonia contains meiotic spermatocytes, followed by the postmeiotic round spermatids and finally elongating spermatids. Mature sperm is released into the lumen. Somatic, testosterone-producing Leydig cells are situated in the interstitial space between the tubules. Spermatogenesis is under a direct control of the hypothalamus-pituitary axis. Gonadotropin-releasing hormone (GnRH) secreted by hypothalamus induces the production of follicle-stimulating hormone (FSH) and luteinizing hormone (LH) in pituitary gland. LH stimulates Leydig cells to produce testosterone (T) that controls spermatogenesis. FSH acts on Sertoli cells inside the seminiferous epithelium. *Early Spc* early spermatocytes, *Late Spc* late spermatocytes, *RS* round spermatids, *ES* elongating spermatids

recognizable from the cell associations (spermatogonia, spermatocytes and spermatids) present in a cross section of the seminiferous tubules (Oakberg 1956). This organization ensures the constant production of high number of spermatozoa during the whole course of sexual maturity.

6.1.2 Regulation of Gene Expression During Spermatogenesis

It has been estimated that 60% of the mouse genome is expressed during testis development from birth to adulthood. Since male germ cell differentiation consists of several unique processes and mechanisms, some of these genes are either male germ cell -specific or testis predominant. Indeed, according to the transcriptome analyses, even 4% of the mouse genome seems to be dedicated to the expression of

genes unique to spermatogenesis (Lee et al. 2009). Gene expression is governed by highly specific regulatory rules in order to control chromatin organization as well as transcriptional events during spermatogenesis (Kimmins et al. 2004; Kimmins and Sassone-Corsi 2005). Spermatocytes and round spermatids are transcriptionally very active, but during the late steps of spermatogenesis, the compaction of sperm chromatin results in a drastic inhibition of transcriptional activity (Tanaka and Baba 2005). Translational control of mRNAs becomes prominent, and after chromatin condensation, gene control is regulated almost exclusively at the posttranscriptional level (Kleene 2003). This is reflected by a high number of RNA-binding proteins in spermatogenic cells, many of them being testis-specific (Paronetto and Sette 2010). Similar to the germline of many different organisms, mammalian male germ cells are characterized by distinct RNA and protein-rich non-membranous cytoplasmic domains called germ granules (Eddy 1970; Chuma et al. 2009). The most prominent of them are the intermitochondrial cement (IMC) in pachytene spermatocytes and the chromatoid body (CB) in round spermatids (Meikar et al. 2011). Given their distinct features and the known protein and RNA composition, germ granules are likely to have a specialized role in mRNA regulation and small RNA-dependent pathways during male germ cell differentiation (Meikar et al. 2011).

6.2 Chromatin Modifications in Male Germ Cells

6.2.1 Chromatin Modifying Proteins

Epigenetic mechanisms play an important part in the control of gene expression programs in male germline. Furthermore, differentiating male germ cells undergo several important epigenetic transitions that are characterized by extensive chromatin alterations (Fig. 6.2). These include the reprogramming of epigenetic marks in PGCs, meiotic chromatin organization and recombination events, and the compaction of haploid genome inside sperm nuclei (Kota and Feil 2010). Therefore, it is not surprising that several chromatin modifying enzymes are expressed during male germ cell differentiation in temporally regulated manner, and their functions are required for the correct development of a male gamete (Godmann et al. 2009). The methylation of cytosine residues of genomic DNA, which takes place predominantly within CpG dinucleotides, is associated with gene silencing, especially in processes such as transposon and repeat satellite sequence silencing and imprinting. Intriguingly, the sperm genome-wide methylation patterns differ markedly from that of somatic cells, thus highlighting the importance of DNA methylation in the control of male germline –specific processes, including the maintenance of unique chromosomal structures in male germ cells (La Salle et al. 2007; Oakes et al. 2007). Five DNA (cytosine-5)-methyltransferases (DNMTs) are characterized in mammals (Trasler 2009). DNMT1 is critical for maintenance of methylation patterns during replication of DNA. DNMT3A and DNMT3B are *de novo* methyltransferases that are highly expressed during embryonic development. DNMT3L does not have

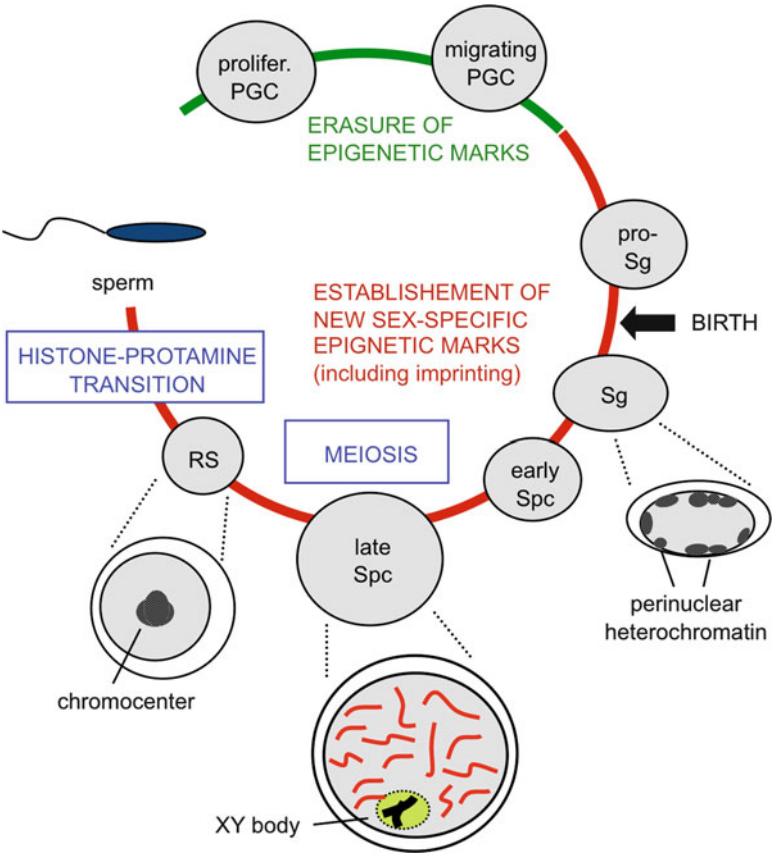

Fig. 6.2 Epigenetic transitions and chromatin dynamics in male germ cells. Germ cell development is accompanied by a massive reprogramming of epigenetic marks. Erasure of the epigenetic marks (indicated with *green line* and *green font*) takes place in PGCs. Novel male specific marks, including the imprinting of certain genes (indicated with *red line* and *red font*) starts in PGCs and continues during the postnatal differentiation. The major epigenetic processes that involve dramatic chromatin dynamics and reorganization, meiosis and chromatin compaction in late spermiogenesis by the histone-protamine transition, are indicated. Special heterochromatin structures involved in gene silencing in male germ cells are highlighted by *drawings*. These structures include the perinuclear heterochromatin in differentiating spermatogonia, the sex body (XY body) in spermatocytes and the chromocenter in spermatids. The details of formation and the purpose of perinuclear heterochromatin in spermatogonia is still obscure. Sex body forms during meiosis when sex chromosomes are condensed and silenced by MSCI. Homologous chromosomes paired by synaptonemal complex are shown in *red* in the nucleus. X and Y chromosomes condensed by heterochromatinization are shown in *black* and sex body is indicated as *green circle*. Chromocenter in the nucleus of round spermatids contains the centromeric heterochromatin of each chromosome, and is thought to serve as an initial organizer of the DNA packing that takes place in the late spermiogenesis

catalytic activity but it functions by interacting with DNMT3A and DNMT3B and modulating their activities. DNMT1, DNMT3A, DNMT3B and DNMT3L are all differentially expressed in germ cell type-specific patterns in both prenatal and postnatal male germ cells (Trasler 2009)

A defined composition of repressive and activating histone modifications ensures proper meiotic and postmeiotic events, but also mediates a highly orchestrated expression of male germ cell –specific genes. Disturbances of the balance between different epigenetic marks usually results in a failure of male germ cell development, as demonstrated by several knockout studies (Godmann et al. 2009). For example, correct methylation patterns on H3 are crucial for normal progression of spermatogenesis, since the deletion H3 methyltransferases has been shown to lead to meiotic problems and male infertility. Double knockout mice for H3 Lysine 9 (H3K9) trimethylases KMT1A and KMT1B (also called SUV39H1 and SUV39H2) and knockout mice for mono/dimethylase KMT1C (also called G9A or EHMT2) show similar meiotic phenotypes even though the mechanisms are presumably different because KMT1A/B are known to catalyze H3K9 methylation associated with heterochromatin, whereas KMT1C acts on euchromatin (Peters et al. 2001; Tachibana et al. 2007). Methylation of H3 at residue K4 is also important, and the deletion of H3K4 histone methyltransferase PRDM9 (also called MEISETZ) results in sterility due to a disrupted spermatogenesis at the meiotic pachytene stage (Hayashi et al. 2005). Likewise, other histone modifications such as acetylation, phosphorylation and ubiquitination, have been demonstrated as essential regulators of spermatogenesis (Kimmins et al. 2007; Kolthur-Seetharam et al. 2009; Roest et al. 1996; Baarends et al. 2003).

6.2.2 Epigenetic Reprogramming

In germ cells, the somatic cell state has to be reprogrammed by genome-wide epigenetic resetting. Reprogramming begins during embryonic development at the time when PGCs migrate and colonize genital ridges (Fig. 6.2). The process begins by near to complete erasure of somatic methylation marks, and is followed by the re-establishment of novel sex-specific DNA methylation patterns, including differential imprinting of the genes in the male and female germ cells (Sasaki and Matsui 2008; Ewen and Koopman 2010). *De novo* methyltransferases DNMT3A and DNMT3B have been demonstrated to be critical for gene imprinting and silencing of repeat sequences in the germline (Kaneda et al. 2004; Kato et al. 2007). While other methyltransferases are expressed also in somatic cells, the expression of the catalytically inactive family member, DNMT3L, is restricted to male and female germ cells. DNMT3L deficiency results in defective methylation of retrotransposons, which induces aberrant transposon expression in the germline (La Salle et al. 2007; Bourc'his and Bestor 2004; Webster et al. 2005). These changes results in severe problems in meiotic progression including widespread nonhomologous synapsis at the pachytene stage. Meiotic defects may be derived from inappropriate alignment

of unmethylated retrotransposon elements, chromosome breaks induced by aberrant retrotransposition events, or altered gene expression. DNA demethylation in PGCs during epigenetic reprogramming is also linked to changes in nuclear architecture, loss of histone modifications, and widespread histone replacement (Seki et al. 2007; Hajkova et al. 2008). The molecular details of massive DNA demethylation was recently clarified by a study demonstrating that the base excision DNA repair pathway provides a mechanism for the demethylation and the extensive chromatin remodeling in the mouse PGCs (Hajkova et al. 2010). Some epigenetic marks remain intact during the epigenetic reprogramming, and thus provide possible means for transgenerational inheritance of epigenome – it is also clear that the period of epigenetic reprogramming is critical for correct maintenance of germline epigenome, and it is especially vulnerable to environmental agents that may induce changes in the epigenome (discussed below).

6.2.3 Epigenetic Control of Meiosis

A central process in gametogenesis is meiosis, which leads to the conversion of diploid cells to haploid gametes. Meiotic events are controlled by epigenetic mechanisms including DNA and histone modifications and chromatin remodeling. Several factors that control histone methylation are essential for meiotic transitions, particularly in the male germline (Nottke et al. 2009). However, on the basis of current knowledge, it is still unclear whether the meiotic problems are caused by the defects in meiosis or for example by indirect effects due to altered gene expression. During meiosis, homologous chromosomes become aligned and form synapsis, which is a prerequisite for the chromosomal crossover and meiotic recombination. Recombination events guarantee that the new organism generated after fertilization is genetically different from its parents. In mammals, crossovers cluster at preferential sites, which are called recombination hotspots. In addition to specific DNA elements, the initiation sites of recombination are enriched with certain epigenetic modifications such as H3K4 trimethylation and H3K9 acetylation (Buard et al. 2009). Specific sequence elements connected to recombination hotspots are recognized by a meiosis-specific H3 methyltransferase PRDM9 (Baudat et al. 2010). Binding of PRDM9 leads to trimethylation of H3K4, which subsequently triggers SPO11-meidated double strand break formation that is required for crossovers. It is important to prevent illegitimate recombination and crossing over at unpaired sites and between nonhomologous chromosomes. A process called meiotic sex chromosome inactivation (MSCI) is utilized to silence unsynapsed sex chromosomes in a chromosomal domain called sex body, and avert the deleterious recombination events during first meiotic prophase (Burgoyne et al. 2009). MSCI and sex body formation are controlled by epigenetic mechanisms, including incorporation of specific histone variants and specific histone modifications, such as phosphorylation, ubiquitination and sumoylation. ATR kinase-mediated phosphorylation of histone variant H2AX at

serine-139 (γH2AX), and the subsequent accumulation of γH2AX is known to be an important signal for triggering MSCI (Burgoyne et al. 2009).

6.2.4 *Postmeiotic Histone Replacement and Chromatin Compaction*

After the two meiotic divisions, the final step of spermatogenesis, spermiogenesis, takes place during which the maturation of spermatids to fully differentiated spermatozoa is completed. Postmeiotic male germ cells undergo a remarkable chromatin remodeling, which enables the extreme compaction of male germ cell nuclei. Chromatin compaction may help to optimize nuclear shape and hence support the ability of sperm cells to swim across the female reproductive tract, and it may also confer additional protection from the effects of genotoxic factors (Kimmins and Sassone-Corsi 2005; Miller et al. 2010). Despite the fundamental nature of this process, the molecular basis of the mechanisms involved remains largely unknown. The major steps in mammals involve the replacement of most of the nucleosomal histones first by transition proteins (TNPs) and subsequently by protamines (PRMs). The timing of TNP and PRM expression is tightly regulated and involves local chromatin changes, including H3K9 demethylation by KDM3A/JHDM2A (Okada et al. 2007). Despite the fact that TNPs constitute even 90% of all chromatin basic proteins after histone removal and before protamines deposition, the exact role of TNPs in histone replacement and chromatin compaction is still obscure. In double knockout mice lacking both TNP1 and TNP2, histones were found to be displaced normally, demonstrating that TNPs do not play a role in the removal of histones. *Tnp1/Tnp2* double knockout spermatids have, however, problems in chromatin condensation (Zhao et al. 2004a, b).

Protamines are small, highly basic arginine-rich proteins that are evolutionarily related to histone H1. The properties of protamines enable over ten times more efficient packing of paternal DNA than what is achieved by histone-built nucleosomes. Mice and humans express two protamines, PRM1 and PRM2. Protamines are essential for the production of spermatozoa, and the haploinsufficiency of either PRM1 or PRM2 disrupts nuclear formation and normal sperm function (Cho et al. 2001). Even though protamine incorporation covers most of the sperm genome, many regions retain nucleosomal histones, which are enriched in specific epigenetic modifications, such as trimethylated H3K4, trimethylated H3K27, and unmethylated DNA. These loci include key developmental genes, imprinted genes, microRNAs and homeotic genes. The specific epigenetic modifications of these genes potentially contribute to the appropriate gene expression during early embryonic development (Kota and Feil 2010; Hammoud et al. 2009; Brykczynska et al. 2010).

The exact mechanisms for histone replacement are still unclear. As discussed below, the incorporation of histone variants into nucleosomes prior to histone-protamine transition is probably involved in creating less stable nucleosomes. In addition, a genome-wide massive histone hyperacetylation, which takes place in

late round spermatids and early elongating spermatids before chromatin compaction, seems to be tightly linked to histone replacement (Hazzouri et al. 2000). A testis-specific bromodomain-containing protein BRDT, which binds acetylated lysines and is implicated in chromatin remodeling, is associated with acetylated histones located in the pericentric regions in elongating spermatids (Govin et al. 2006). Functional BRDT is required for the normal elongation of spermatids, suggesting that it may be involved in acetylation-mediated events during chromatin compaction (Shang et al. 2007). Interestingly, ubiquitination of H2A and H2B by the E3 ubiquitin ligase RNF8 has been demonstrated to be an important signal in promoting H4 hyperacetylation in elongating spermatids (Lu et al. 2010). Disruption of *Rnf8* gene in mice disrupts the H4 hyperacetylation and the mice are deficient in global nucleosome removal (Lu et al. 2010).

6.2.5 Histone Variants

Before the replacement of almost all the histones by protamines, the male germ cell genome is organized by the replacement of canonical histones by a variety of histone variants (Kimmins and Sassone-Corsi 2005; Gaucher et al. 2010; Boussouar et al. 2008). A very high number of both core and linker histone variants are expressed in male germ cells, many of them being testis-specific. The large scale histone replacement starts already very early during embryonic germ cell development, as demonstrated by a study revealing the involvement of histone displacement in the erasure and re-establishment of DNA methylation in PGCs (Hajkova et al. 2008). However, the most active nucleosome re-organization by histone variants takes place during meiotic and postmeiotic differentiation. During meiosis, the formation of sex body in pachytene spermatocytes is accompanied by the incorporation of specific histone variants such as H3.3 and macroH2A. Histone variants are suggested to create less stable nucleosomes, thus a large-scale incorporation of histone variants in haploid cells serves as a potential mechanism for histone replacement by protamines. It has been hypothesized that the incorporated histone variants form chromatin domains with unstable nucleosomes, which may then constitute preferential targets for nucleosome disassembly and histone displacement (Gaucher et al. 2010). A region-specific assembly of histone variants in specific chromosomal domains has been reported in spermatids. Two H2A-like variants H2AL1 and H2AL2 are synthesized in elongating spermatids and become specifically associated with pericentric regions just before and during the assembly of protamines, thus linking these variants to the differential organization of pericentric heterochromatin during mouse spermiogenesis (Govin et al. 2007). A testis-specific linker histone variant H1T2 associates with chromatin domains localized at the apical pole of the nucleus of late round and elongating spermatids, and is thus involved in creating a polarity in the spermatid nucleus (Martianov et al. 2005). Mice deficient for H1T2 show impaired chromatin compaction resulting in reduced fertility (Martianov et al. 2005).

6.3 Small RNAs in Male Germ Cells

6.3.1 Introduction to Male Germline Small RNAs

There are at least three different populations of small RNAs in mammalian testis: microRNA (miRNAs), endogenous small interfering RNAs (endo-siRNAs) and PIWI-interacting RNAs (piRNAs) (Fig. 6.3). The miRNA pathway is a well-understood posttranscriptional silencing mechanism. miRNAs precursors are endogenous long hairpin loop primary transcripts, which are processed in the nucleus by double-stranded RNA endonuclease Drosha into pre-miRNAs. In the cytoplasm, the pre-miRNAs become targets for another endonuclease, Dicer, which cuts them into short (usually 21 nt) double-stranded miRNAs. Eventually one strand

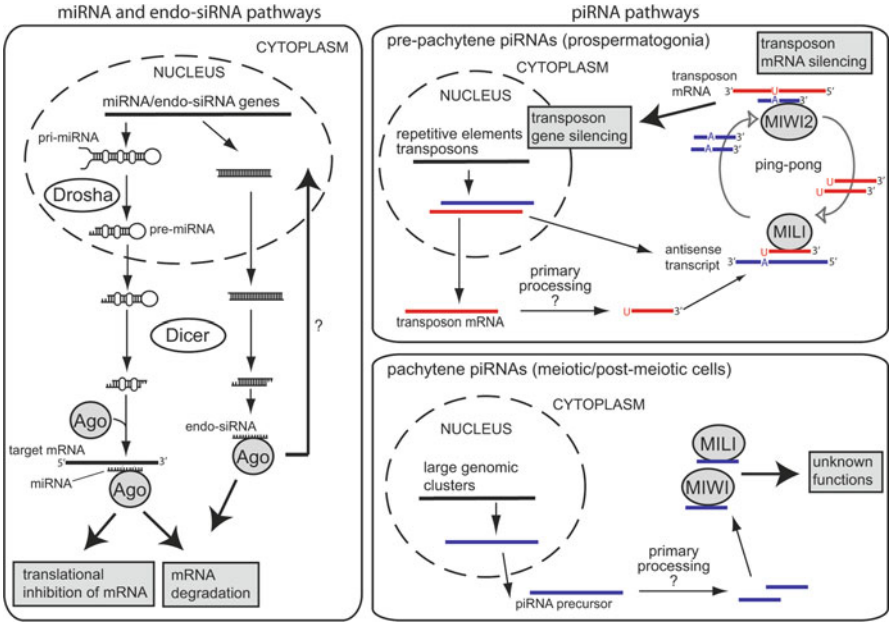

Fig. 6.3 Biosynthesis and functions of small RNAs in mammalian male germline. miRNAs are produced from long imperfect hairpin loops and cut successively by Drosha and Dicer into mature miRNAs. Endo-siRNAs are processed by Dicer from double-stranded RNA precursors. miRNAs and siRNAs form RNA-induced silencing complex (RISC) together with AGO proteins, and regulate the stability or translation of their target mRNAs. Other functions, including the epigenetic chromatin modification in the nucleus, have also been suggested. The production of MILI and MIWI2-bound pre-pachytene piRNAs is explained by the so-called ping-pong mechanism where piRNAs from the opposite strand are promoting each others synthesis in a feed-forward way. Pre-pachytene piRNAs silence transposons posttranscriptionally as well as at the chromatin level through methylation-dependent mechanisms. The primary piRNAs that start the ping-pong cycle are cut out from the transposon mRNAs by an unknown mechanism. Pachytene piRNAs bind MIWI and MILI and are produced in vast quantities presumably from long single-stranded piRNA precursors. The details of their production mechanisms or functions are not yet characterized

of the processed mature miRNA is loaded onto AGO subfamily member of Argonaute proteins in the RNA induced silencing complex (RISC), where it guides the recognition of target mRNAs by imperfect base-pairing leading to mRNA translational inhibition or decay (Lee et al. 2004; Krol et al. 2010). The predominant function of mammalian miRNAs has been reported to be the destabilization of their target mRNAs (Guo et al. 2010). Mature siRNAs are similar to mature miRNAs, but they are fully complementary with their targets and result in the target RNA cleavage. siRNAs derive from long dsRNA precursors and their synthesis is Drosha-independent and requires only Dicer activity. siRNA precursors are usually introduced in cells exogenously, for example by viruses, but as originally described in plants and nematodes, also endogenous siRNAs (endo-siRNAs) are produced and participate in gene silencing (Lau 2010). Moreover, there is increasing evidence that endo-siRNAs can be used as a gene control mechanism in mammals (Lau 2010; Tam et al. 2008; Watanabe et al. 2008; Song et al. 2011).

In contrast to miRNAs that are widely expressed in different tissues, piRNAs are predominantly expressed in the germline and are currently mostly known by their role in the silencing of transposable elements that become activated during DNA demethylation in PGCs (Siomi et al. 2011). piRNAs are named by their direct association with the PIWI proteins (MILI, MIWI and MIWI2 in mice), that belong to evolutionally conserved Argonaute protein family together with AGO proteins (Cenik and Zamore 2011). piRNAs were first identified either by sequencing the characteristic ~30-nt band from total testis RNA extracts or by sequencing the small RNAs that co-immunoprecipitated with PIWI proteins in mouse testis (Aravin et al. 2006; Girard et al. 2006; Grivna et al. 2006; Watanabe et al. 2006). piRNAs are highly abundant in mammalian testis, and after several deep sequencing experiments, the suggested amount of different piRNAs is in hundreds of thousands (Aravin et al. 2007a). Processing of piRNAs does not involve Drosha or Dicer, and is thus separate from miRNA and siRNA biogenesis. Careful analysis of piRNAs that associate with different PIWI proteins has revealed at least two distinguishable classes of piRNAs in mammalian testis which are named by their expression time as pre-pachytene and pachytene piRNAs. Although similar at the molecular level, they have different tasks and mechanism of action, they associate with different PIWI proteins and derive from different regions of the genome.

6.3.2 Control of Spermatogenesis by miRNAs and Endogenous siRNAs

Components of miRNA machinery are found in the male germline and there are several miRNAs that are expressed either specifically or predominantly in the testis (Ro et al. 2007; Chiang et al. 2010; Gonzalez-Gonzalez et al. 2008; Kotaja et al. 2006; Korhonen et al. 2011). Interestingly, many of the testis-expressed miRNA genes are located in miRNA gene clusters on the X chromosome where they are able to escape from the transcriptional silencing of sex chromatin during meiosis by an unknown mechanism (Song et al. 2009). The importance of Dicer in mouse female and male germ cell maturation has been demonstrated by studies analysing different conditional

tissue-specific knockout mouse lines (Korhonen et al. 2011; Murchison et al. 2007; Tang et al. 2007; Hayashi et al. 2008; Maatouk et al. 2008). In mouse testis, Sertoli cell–specific deletion of *Dicer1* revealed its crucial importance for the normal function of these somatic nursing cells in supporting male germ cell differentiation (Papaioannou et al. 2009, 2010). Knockout mice with spermatogonia-specific deletion of *Dicer1* demonstrated the imperative role of Dicer and Dicer-dependent small RNAs in the regulation of postnatal male germ cell development (Korhonen et al. 2011). The most prominent defects in *Dicer1*-depleted spermatids were found during haploid differentiation, especially in the elongation of nuclei and the organization of chromatin. The molecular mechanisms that are used by Dicer-dependent pathways to control spermatogenesis are still unclear. Posttranscriptional control is of central importance during haploid differentiation due to the transcriptional silencing that results from the tight packing of chromatin with protamines (Kimmins and Sassone-Corsi 2005; Gaucher et al. 2010). Since the most striking defects in the differentiation of Dicer-null male germ cells coincide chronologically with chromatin condensation and transcriptional silencing, it can be envisaged that Dicer is involved in this post-transcriptional control of haploid mRNAs.

Dicer is involved also in the production of endo-siRNAs in male germline, which emphasizes the diversity of Dicer-dependent pathways (Song et al. 2011). Endo-siRNA pathways were originally thought to be absent in mammalian cells due to an aggressive innate immune response to intracellular dsRNAs that is indicative of a viral infection (interferon response) (Sen and Sarkar 2007). Germ cells are notable exceptions since they do not activate such a response when injected with dsRNA (Svoboda et al. 2000). Male germ cell endo-siRNAs have been reported to function in posttranscriptional control of a wide variety of protein encoding mRNAs, but other functions, possibly related to chromatin modifications, are also suggested (Song et al. 2011). Evidence of a small RNA–mediated transcriptional gene silencing and regulation of heterochromatin formation and maintenance is emerging, even though the mechanistic aspects still remain unclear (Moazed 2009). Possible nuclear functions of Dicer are supported by its localization in the nucleus and specifically on certain chromosomal domains (Khalil and Driscoll 2010; Sinkkonen et al. 2010), and its involvement in the centromeric repeat transcript silencing in mouse male germ cells (Korhonen et al. 2011) and in mouse embryonic stem cells (Kanellopoulou et al. 2005; Murchison et al. 2005). However, further studies will be required to reveal the mechanistic connection of Dicer and Dicer-dependent small RNAs with heterochromatin formation, regulation of repeat-derived transcripts and control of chromatin organization in differentiating male germ cells.

6.3.3 Pre-Pachytene piRNAs as Regulators of Transposon Expression

Each PIWI protein is expressed at a different timeframe during male germ cell development and binds a different subset of piRNAs. MIWI2 and MILI expression starts in fetal prospermatogonia, which are undergoing the genome-wide

reorganization of DNA methylation pattern to generate novel gametic epigenetic marks (Aravin et al. 2008). During that time, everything which is otherwise epigenetically silenced becomes derepressed, so the cells need an alternative mechanism to protect their genome against the invasion of activated transposable elements. When analysing the MILI- and MIWI2-bound piRNAs from the total piRNA pool, a strikingly uniform subclass of piRNAs emerged that originate from repeat sequences related to transposable elements and heterochromatic regions. They were called pre-pachytene piRNAs and they are now the most studied and understood piRNAs, although they represent only a tiny fraction of all mammalian piRNAs. MILI and MIWI2 together with pre-pachytene piRNAs participate in silencing of transposable elements both at epigenetic and posttranscriptional level in fetal and neonatal germ cells (Aravin et al. 2007a, 2008; Carmell et al. 2007; Kuramochi-Miyagawa et al. 2008). In the knock-out mice of either of these proteins the transposons are uncontrollably expressed, causing damage in the genome integrity of the cell, which eventually leads to meiotic arrest and sterility (Carmell et al. 2007; Kuramochi-Miyagawa et al. 2004).

Analysis of the piRNA sequences and timing of their expression has led to the model of piRNA biogenesis mechanism, called the ping-pong cycle, that is mediated by PIWI proteins (Fig. 6.3) (Aravin et al. 2008; Brennecke et al. 2007). By this model, the transposon mRNAs activate the amplification of their target piRNAs by sense-antisense RNA amplification loop. In mouse, the transposon transcripts themselves become the substrates of so-called primary piRNAs by a yet unknown primary piRNA processing pathway. These transposon-derived sense primary piRNAs are bound to MILI and mediate the production of the secondary piRNAs by cleaving the antisense transposon transcripts that are generated genetically from piRNA clusters. The secondary piRNAs pair with MIWI2 and in turn, target the respective transposon mRNAs, which becomes the substrates of next piRNA molecules. This amplification loop is triggered and regulated by the presence of transposon mRNAs. The primary piRNAs share a 5′ U-bias, which corresponds to the bias of 10A in the complementary secondary piRNAs. This footprint is characteristic to the ping-pong cycle in different organisms where it acts as an adaptive immune system that optimizes the piRNA population accordingly to the target elements.

6.3.4 Pachytene piRNAs and the Chromatoid Body

Pachytene piRNAs arise in spermatocytes during meiosis around day 14 post partum, peak in haploid round spermatids and disappear during later steps of spermiogenesis, overlapping the expression of their binding PIWI partners, MILI and MIWI. The amount of pachytene piRNAs per each pachytene spermatocyte or round spermatid is remarkable as it is possible to visualize the characteristic 30-nucleotide band in total testis RNA extract just in ethidium bromide or SybrGold stained polyacrylamide gel (Aravin et al. 2006; Girard et al. 2006; Grivna et al. 2006). The amount of pachytene piRNAs in cells is suggested to be around a million molecules, but the individual copy number is low, which means that piRNA

population is very heterogeneous consisting of hundreds of thousands of different piRNAs (Aravin et al. 2006, 2007b). Compared to their pre-pachytene counterparts, the biogenesis and function of pachytene piRNAs is unknown. They share the preference to 5′ U, but lack the 10A preference and do not produce homologous antisense transcripts, which is characteristic to the ping-pong cycle. Pachytene piRNAs are also devoid of sequences relative to active transposons. Instead they map into large sparse clusters, from tens to hundreds of kilobases along the genome with most of the clusters being derived from one of the two genomic strands (Aravin et al. 2007a, b). There is very little conservation of individual piRNA sequences between different mammals, but surprisingly there is a significant conservation of the genomic locations of mammalian piRNA clusters (Betel et al. 2007). Interestingly, pachytene piRNAs in round spermatid are concentrated in chromatoid bodies – male germ cell-specific cytoplasmic ribonucleoprotein germ granules of remarkable size and peculiar features (Meikar et al. 2010, 2011; Kotaja and Sassone-Corsi 2007). As the chromatoid body also concentrates MIWI, RNA binding proteins and helicases in addition to longer polyadenylated RNAs (Kotaja et al. 2006; Meikar et al. 2010), it is tempting to speculate that it serves as a processing centre for pachytene piRNAs and mRNAs.

6.4 Implications to Human Diseases

6.4.1 Epigenetic Transgenerational Inheritance

Epigenetic status of the chromatin is mitotically stable – as the cell undergoes mitosis the epigenome is replicated. Germline cells have the capacity of erasing the epigenetic memory and resetting the epigenome. Certain marks, however, appear to escape the reprogramming and can be transmitted to the offspring, which enables transgenerational epigenetic inheritance. Because the environmentally induced changes in the epigenome of the germline becomes permanently programmed and the altered epigenome and phenotype can be transmitted to subsequent progeny, environmental epigenetics has certainly a critical role in disease etiology (Walker and Gore 2011; Skinner 2011). Environmental factors, such as endocrine disrupting chemicals (EDCs) that interfere with the ability of endocrine systems to maintain homeostasis, are able to influence the epigenome. The critical time for environmental exposure is the period when the germline cell fate is determined and epigenome is reprogrammed in mammalian fetus. If the mother is exposed to environmental agents such as EDCs during this time, the germline epigenome of male offspring can be modified and permanently altered. Studies in mice have demonstrated the transgenerational inheritance of the epigenetic changes in DNA methylation caused by the exposure of embryos to vinclozolin, diethylstilbesterol, bisphenol A and polychlorinated biphenyls that are representative EDCs (Walker and Gore 2011; Skinner 2011). DNA methylation has been proposed as a major mechanism for transgenerational epigenetic effects because of vast changes of DNA methylation

patterns during early mammalian development (Lange and Schneider 2010). Since piRNAs in mammalian PGCs have been implicated in the methylation of transposable elements upstream of DNMT, it has been suggested that small RNAs could mediate epigenetic inheritance through homology-dependent silencing systems (Suter and Martin 2010).

6.4.2 Epigenetic Control of Germline Antigens in Cancer

Cancer germline (CG) -antigens are classified as factors normally expressed only by germ cells, but found to be aberrantly expressed in a wide range of human cancers. Epigenetic status of germline cells are programmed to repress somatic genes and support germline differentiation. Cancer cells undergo large-scale epigenome alterations, which may lead to aberrant activation of the CG genes that are normally silenced outside the germline (Wang et al. 2011). Recent evidence suggests epigenetic mechanisms, particularly DNA methylation, as primary regulators of CG gene expression in normal and cancer cells. The mechanisms by which CG antigens promote tumour growth are still unclear, but it has been proposed that the off-context activity of some germline epigenome regulators could reprogram the somatic epigenome toward a malignant state by favouring self-renewal and sustaining cell proliferation, and in this way to support oncogenic properties of the cells (Wang et al. 2011). There has been a search for good tumour antigens in the past 100 years. An ideal cancer antigen for immunotherapy would be specifically expressed in tumours, absent from healthy tissue and critical for the survival of cancer. Importantly, CG-antigens have potentially a high clinical relevance in cancer immunotherapy because they are immunogenic and because of their restricted expression pattern in physiological conditions (Simpson et al. 2005; Akers et al. 2010).

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Chapter 7
Epigenetic Regulation of Skeletal Muscle Development and Differentiation

Narendra Bharathy, Belinda Mei Tze Ling, and Reshma Taneja

Abstract Skeletal muscle cells have served as a paradigm for understanding mechanisms leading to cellular differentiation. Formation of skeletal muscle involves a series of steps in which cells are commited towards the myogenic lineage, undergo expansion to give rise to myoblasts that differentiate into multinucleated myotubes, and mature to form adult muscle fibers. The commitment, proliferation, and differentiation of progenitor cells involve both genetic and epigenetic changes that culminate in alterations in gene expression. Members of the Myogenic regulatory factor (MRF), as well as the Myocyte Enhancer Factor (MEF2) families control distinct steps of skeletal muscle proliferation and differentiation. In addition, growing evidence indicates that chromatin modifying enzymes and remodeling complexes epigenetically reprogram muscle promoters at various stages that preclude or promote MRF and MEF2 activites. Among these, histone deacetylases (HDACs), histone acetyltransferases (HATs), histone methyltransferases (HMTs) and SWI/SNF complexes alter chromatin structure through post-translational modifications to impact MRF and MEF2 activities. With such new and emerging knowledge, we are beginning to develop a true molecular understanding of the mechanisms by which skeletal muscle development and differentiation is regulated. Elucidation of the mechanisms by which epigenetic regulators control myogenesis will likely provide a new foundation for the development of novel therapeutic drugs for muscle

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dystrophies, ageing-related regeneration defects that occur due to altered proliferation and differentiation, and other malignancies.

7.1 Introduction

7.1.1 Skeletal Myogenesis in the Embryo and the Adult

All skeletal muscle of the vertebrate body and some head muscles are derived from somites, that are laid down on each side of the neural tube and notochord during embryogenesis (Buckingham 2001; Cossu et al. 1996a; Pownall et al. 2002; Tajbakhsh and Cossu 1997). Cells in the dorsal portion of the somite form the dermomyotome, which gives rise to the dermis and most skeletal muscles of the body. Through inductive and repressive signals from surrounding tissues, somites acquire dorsal-ventral, anterior-posterior, and medial-lateral polarity. In response to signals from the notochord such as Wnt and Sonic Hedgehog, the expression of the paired homeobox transcription factors Pax3 and Pax7 are induced in muscle progenitor cells in the dermamyotome resulting in the specification of muscle cells. Pax3 induces the expression of the myogenic regulatory factors Myf5, and consequently MyoD, commiting cells to the myogenic lineage. Myf5 and MyoD establish two distinct populations of cells, that give rise to the epaxial and hypaxial muscles (Ordahl and Le Douarin 1992; Cossu et al. 1996b). Differentiation of muscle cells is subsequently mediated by MyoD, Myogenin and MRF4.

Satellite cells, the local muscle stem cells, arise from Pax3 and Pax7 expressing muscle progenitor cells that originate in the dermomyotome (Relaix et al. 2005; Gros et al. 2005). In the adult muscle, satellite cells are located between the sarcolemma and basal lamina of the muscle fiber, and are the main source of myogenic precursors in the adult. The expression of Pax7 is required for the maintenance of satellite cells and generation of committed progenitors (Relaix et al. 2006; Kuang et al. 2006). Pax7 binds to the Myf5 promoter and recruits the HMT complex Wdr5–Ash2L–MLL2 (McKinnell et al. 2008) that directs trimethylation of histone H3 lysine 4 (H3K4me3). Methylation of H3K4 marks chromatin in a conformation permissive for transcription resulting in the upregulation of Myf5 expression. Thus, Pax7 induces chromatin modifications that stimulate transcriptional activation of Myf5 and thereby regulates commitment into the myogenic developmental programme. Upon muscle injury, quiescent satellite cells are activated and undergo proliferation to give rise to myoblasts. Activated satellite cells expressing Pax7 and MyoD undergo several rounds of proliferation, and subsequently upregulate myogenin and MRF4 to differentiate and form new myofibers repairing the damaged muscles. The onset of differentiation is preceded by down-regulation of Pax7. A distinct population of satellite

cells retain Pax7 expression but dowregulate MyoD, reversibly exit the cycle, and relocate to the basal lamina, thereby replenishing the satellite cell pool (Zammit et al. 2004).

7.2 Transcriptional Control of Myogenesis

Differentiation of skeletal myoblasts is regulated by two classes of transcription factors – the Myogenic regulatory factors (MRF) that include MyoD, Myf5, MRF4 and Myogenin; and the Myocyte Enhancer Factor (MEF2) family which includes MEF2-A, -B, -C and -D (Molkentin and Olson 1996; Black and Olson 1998). A landmark discovery that facilitated the understanding of the genetic control of skeletal muscle differentiation was the identification of MyoD as a master regulator of myogenesis through its ability to convert cultured fibroblasts cells into skeletal muscle (Davis et al. 1987). MyoD expression is selectively restricted to skeletal muscle cells. In non-muscle cells MyoD expression is repressed by DNA methylation, an epigenetic modification mediated by DNA methyltransferases (DNMTs). Thus, treatment with the demethylating agent 5-azacytidine derepresses the MyoD promoter and induces the differentiation of fibroblasts into muscle cells (Lassar et al. 1986). Once induced in committed myoblasts, MyoD expression promotes cell cycle exit by regulation of p21 expression (Halevy et al. 1995) as well as expression of myogenin (Fig. 7.1). Terminal differentiation is characterized by the expression

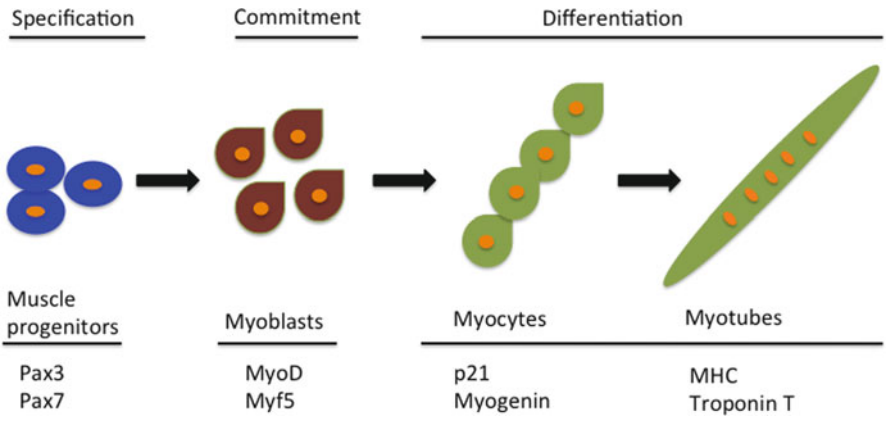

Fig. 7.1 Schematic representation of skeletal muscle differentiation. Pax3/Pax7 positive precursor cells arising from the dermamyotome initiate expression of Myf5 and MyoD and undergo commitment towards the myogenic lineage. MyoD and Myf5 expressing cells undergo expansion and proliferate to give rise to myoblasts. Upon appropriate differentiation cues, myoblasts irreversibly exit the cell cycle and myocytes express early differentiation markers p21 and Myogenin. At later stages, terminally differentiated multinucleated myotubes are characterized by expression of myosin heavy chain (MHC) and Troponin T

of late differentiation markers MHC and Troponin T. Subsequently, three other members of the MRF gene family were identified: Myogenin, Myf5 and MRF4. While all MRFs share the property of converting non-muscle cells to the myogenic lineage, they are expressed at different times during embryonic development. Myf5 is the first MRF to be expressed in the somite followed by MyoD in undifferentiated proliferating myoblasts (Buckingham 1992), whereas Myogenin and MRF4 are activated during differentiation. This led to hypothesis that Myf5 and MyoD are required for determination of myogenic precursor cells whereas myogenin and MRF4 are required for terminal differentiation. Disruption of MyoD or Myf5 alone in mice results in a delay in the formation of hypaxial and epaxial muscles respectively, with no gross defects in muscle differentiation due to functional redundancy between the two genes (Rudnicki et al. 1992; Braun et al. 1992). However, loss of both MyoD and Myf5 in mice results in reduced muscle masses consequent to a defect in the formation of myoblasts (Rudnicki et al. 1993). In the absence of myogenin, early steps of myogenesis occur normally. However the formation of myofibers is impaired and myogenin mutants die perinatally with an absence of differentiated muscles (Hasty et al. 1993; Nabeshima et al. 1993; Venuti et al. 1995). Loss of MRF4 alone results in subtle defects in myogenesis resulting in a slight reduction of muscle specific genes but no overt defect in muscle development (Olson et al. 1996). The phenotype of single and double MRF mutants indicate a genetic pathway of MRF function with some functions that are overlapping between MyoD and Myf5, whereas some are unique. It is unclear whether these differences are due to inherent functional differences, or are mainly caused by different expression patterns and/or transcriptional activation of MRFs. The MEF2 genes are expressed at very low levels in myoblasts and are induced during differentiation by MRFs. Once induced, Myogenin and MEF2C reside in a positive feedback loop enhancing each others transcription, with MEF2C being essential for the correct spatio-temporal expression of myogenin (Dodou et al. 2003; Cserjesi and Olson 1991). Together, MRFs and MEF2 proteins create a positive feedback loop and also regulate transcription of muscle structural genes.

7.3 Regulation of MyoD and MEF2 Activities

All MRFs and MEF2 family members contain DNA-binding and dimerization domains. MRFs dimerize with the ubiquitously expressed bHLH E proteins E12 and E47 (two splice variants of the E2A gene). The MRF-E protein heterodimers bind to their target E-box (CANNTG) site through the basic region to activate expression of downstream targets. The helix-loop-helix (HLH) domain in MRFs is required for dimerization with E proteins. In addition, MRFs interact with MEF2 through their basic domain. All MEF2 factors bind to A/T rich regions in muscle regulatory promoters and share the MADS box domain that mediates dimerization and DNA binding, and a MEF2 domain that mediates co-factor recruitment. Unlike MRFs, MEF2 factors do not possess myogenic activity on their own. However,

they are able to potentiate myogenesis induced by MRFs, and increase efficiency of conversion of non-muscle cells (Molkentin et al. 1995).

Since MyoD and MEF2 factors are expressed in myoblasts, several regulatory mechanisms ensure that their activity is tightly regulated until appropriate cues are present that are permissive for differentiation. For instance, MyoD expression and activity in myoblasts is controlled through several mechanisms including the presence of Inhibitor of differentiation (Id) proteins that sequester E proteins from MyoD; as well as other inhibitors such as Twist, Mist1, MyoR, and Sharp-1 that inhibit MyoD transcriptional activity, DNA-binding, and dimerization with E-proteins (Benezra et al. 1990; Spicer et al. 1996; Lu et al. 1999; Lemercier et al. 1998; Azmi et al. 2004). During differentiation, the expression levels of many of these inhibitory molecules including Id1 decline allowing for increased MyoD transcriptional activity. In addition, the transcriptional network that regulates myogenesis, a number of key epigenetic marks at muscle specific loci are altered during the commitment, proliferation and differentiation stages. Post-translational modifications of histone tails mediated by HDACs, HATs and HMTs alter the chromatin configuration allowing for a transcriptional control of MRF and MEF2 activity in undifferentiated and differentiated cells (McKinsey et al. 2001; Guasconi and Puri 2009).

7.4 Chromatin Modifications in Undifferentiated Myoblasts

MyoD is expressed in committed myogenic precursor cells and has the ability to initiate the differentiation program. The premature activation of differentiation is prevented by several epigenetic mechanisms including recruitment of HDACs and HMTs which restrain MyoD and MEF2 activities in myoblasts (Fig. 7.2). Histone deacetylases are divided into three categories based on their homology to yeast proteins Rpd3p (class I), Hda1p (class II), and Sir2p (class III) (de Ruijter et al. 2003; North and Verdin 2004). While class I and II HDACs are sensitive to TSA, class III HDACs are not, and the deacetylase activity of class III enzymes relies on the cofactor NAD. All three classes of HDACs are involved in preventing premature myogenesis and function to regulate MyoD and MEF2 activities, as well as in sensing the redox balance. HDAC1, HDAC4/5 and SirT1 interact with MyoD and MEF2 factors and function not only deactylate histones but also deactylate transcription factors. In undifferentiated myoblasts, HDAC1 preferentially associates with MyoD in myoblasts through its bHLH domain. This association results in local deacetylation of histones resulting in a transcriptionally repressive chromatin configuration. Overexpression of HDAC1 inhibits muscle differentiation and is associated with reduced histone acetylation on late muscle promoters MCK and MHC (Puri et al. 2001; Mal et al. 2001). In addition, HDAC1 can deacetylate MyoD in vitro, that may additionally contribute to keeping MyoD inactive (Mal and Harter 2003; Mal et al. 2001). On the other hand, MEF2 activity in myoblasts is negatively regulated by HDAC4 and HDAC5. MEF2 factors interact directly with HDAC 4/5 in resulting in repression of MEF2-dependent transcription (Lu et al. 2000; McKinsey et al. 2001).

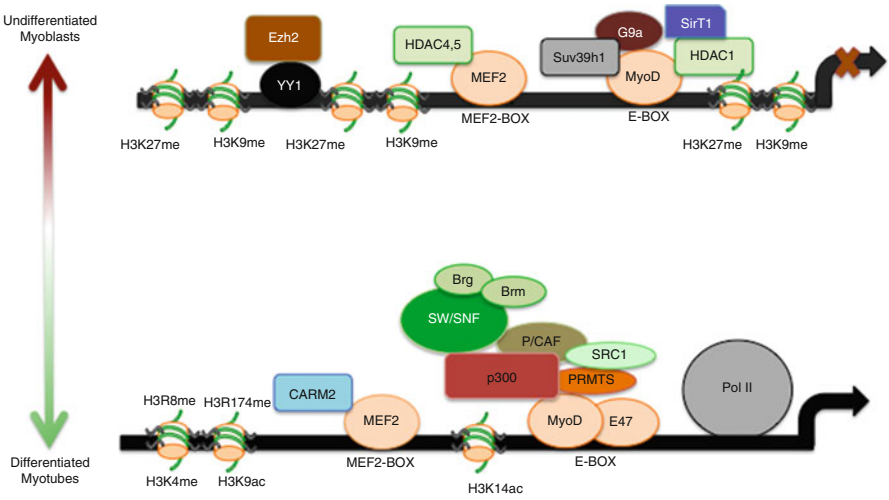

Fig. 7.2 Epigenetic reprogramming at muscle promoters in undifferentiated myoblasts and differentiated myotubes. In undifferentiated myoblasts, MyoD interacts with HDAC1 and SirT1, as well as HMTs Suv39h1 and G9a. Ezh2 is recruited on muscle promoters by YY1. MEF2 interacts with HDAC4/5. HMTs and HDACs result in local repressive chromatin marks such as H3K9me, H3K27me and deacetylation of histone tails. In differentiated cells, recruitment of HATs and chromatin remodelling enzymes by MyoD and MEF2 permits extensive reprograming of muscle promoters resulting H3K9 and H3K14 acetylation and H3K4, H3R8 and H3R17 methylation

Consistently, overexpression of HDAC4 or HDAC5 inhibits differentiation and inhibits both early and late muscle differentiation genes. The Class III HDAC SirT1, whose activity is regulated by the availability of NAD^+, forms a complex with MyoD and p300/CBP associated factor (P/CAF). Overexpression of SirT1 inhibits MyoD activity in myoblasts and thereby myogenesis (Fulco et al. 2003). During differentiation, the $NAD^+/NADH^+$ ratio decreases resulting in reduced SirT1 activity and allowing P/CAF to acetylate histones and MyoD (see below). Thus SirT1 functions as a redox sensor responding to metabolic changes that occur during differentiation.

In addition to HDACs, which mediate deacetylation of histones in undifferentiated cells, the SET-domain containing HMTs are critical mediators of muscle gene repression. In myoblasts, high levels of Histone H3 lysine 9 methylation (H3K9me) which is associated with gene repression, is apparent on the early myogenic promoters myogenin (Zhang et al. 2002; Mal and Harter 2003). The Su(var)3-9 family molecules were the first H3K9 methyltransferases to be described (Rea et al. 2000) and subsequently many others have been characterized. Almost all of them contain a SET domain and include Suv39h1, 2; G9a, GLP/Eu-HMT1; SETDB1, SETDB2; RIZ1/PRDM2; KYP/SUVH4; and DIM-5. Among these, Suv39h1 is the principal enzyme responsible for accumulation of H3K9me3 and is enriched in heterochromatin. Suv39h1 interacts with MyoD in undifferentiated myoblasts and inhibits its

activity and myogenic differentiation (Mal 2006). H3K9me also serves as a platform for recruitment of the heterochromatin protein 1 (HP1) that leads to stable repression via formation of a heterochromatic structure. Similar to Suv39h1, the euchromatic methyltransferase G9a, which is mainly responsible for mono- and dimethylation of H3K9, is also expressed in undifferentiated myoblasts and declines upon myogenic differentiation. Interestingly, G9a mediates H3K9me2 on the myogenin promoter as well as methylates MyoD to control its activity (Ling et al. 2012). Thus it is possible that G9a and Suv39h1 serve to functionally maintain an undifferentiated state by impacting H3K9me on distinct promoters, or may act in sequence to mediate and maintain facultative and stable repression. A second repressive mark seen on late myogenic promoters in undifferentiated cells is H3K27me3 that is mediated by Ezh2, which forms the catalytic unit of Polycomb repressor complex (Caretti et al. 2004). Ezh2 is recruited on late myogenic promoters through the transcription factor YY1 and is found in complexes with HDAC1.

7.5 Chromatin Modifications and Remodelling During Differentiation

One of the early requirements during differentiation is an irreversible exit of myoblasts from the cell cycle. The retinoblastoma protein Rb plays a key role in cell cycle arrest of myoblasts (Huh et al. 2004). Interestingly, HDAC1, which inhibits MyoD in myoblasts, associates with Rb during differentiation. The Rb-HDAC1 complex represses E2F target genes and thereby silences S-phase genes (Puri et al. 2001; Blais et al. 2007). In addition, Suv39h1, which mediates H3K9me, has been shown to silence proliferation genes. These findings are however somewhat at odds with its proposed role as an inhibitor of MyoD activity and muscle differentiation (Ait-Si-Ali et al. 2004; Mal 2006). Finally, Mixed lineage leukemia 5 (MLL5) which is upregulated during quiescence also suppresses S-phase genes including the cyclin A2 promoter (Sebastian et al. 2009). Thus MLL5 may be important in maintaining satellite cell quiescence and expression of muscle determination genes.

The onset of differentiation also requires extensive reprogamming at muscle specific promoters and the replacement of repressive chromatin configuration with those associated with activation. During differentiation, deacetylation of histones H3 and H4, and methylation of H3K9 and H3K27 mediated by HDAC1, HDACII, and HMTs are erased, that correlate with a decline in the expression of Suv39h1, G9a, Ezh2 and HDAC1. Moreover, HDAC4/5 are exported from the nucleus by a CaMK-dependent mechanism during differentiation, resulting in a loss of HDAC4/5 and MEF2 interaction, allowing for a derepression of MEF2 activity (McKinsey et al. 2001).

Conversion of MyoD from a transcriptionally inactive state in myoblasts to an active state during differentiation is contigent upon its activation by several chromatin modifiers and remodeling proteins. These include CBP/p300, P/CAF, the arginine methyltransferase Carm1/Prmt4, Prmt5, and the ATPase dependent SWI/SNF

remodeling complexes that are recruited to muscle promoters. PCAF and p300/CBP form a multimeric complex with MyoD and are required for MyoD to promote myogenic differentiation. p300/CBP acetylate histones H3 and H4, followed by acetylation of MyoD by P/CAF (Puri et al. 1997; Sartorelli et al. 1999; Dilworth et al. 2004). Acetylation of MyoD by P/CAF occurs at three lysine residues K99, K102 and K104 in its basic DNA-binding domain, that stimulates MyoD DNA-binding. Activation of MyoD results in myogenin expression, and a replacement of H3K9me3 by the transcriptionally permissive H3K4me3 on the myogenin promoter (Rampalli et al. 2007).

The SWI/SNF chromatin remodelling complexes also play an essential part in activation of the muscle differentiation program. Brg1, the ATPase subunit of SWI/SNF, is required for nucleosome remodeling and correlates with the presence of Pol II holoenzyme on muscle promoters. SWI/SNF recruitment is dependent on p38 activity, thus linking extracellular signals to nucleosome remodelling (Simone et al. 2004; de la Serna et al. 2005; Albini and Puri 2010).

Carm1/Prmt4, a type I methyltransferase interacts with MEF2 proteins and results in dimethylation of H3R17 (H3R17me2), whereas Prmt5, a type II arginine methyltransferase, associates with MyoD and methylates H3R8 (Chen et al. 2002; Dacwag et al. 2009). Both Carm1/Prmt4 and Prmt5 proteins have been demonstrated to be required at distinct steps of skeletal myogenesis. Chromatin IP experiments have revealed that Carm1/Prmt4 and H3R17me2 are present on the promoters of late myogenic regulatory regions such as MCK. On the other hand, Prmt5 and H3R8me2 binding is apparent on both early (myogenin) and late (MCK, dystrophin) myogenic promoters although Prmt5 appears to be dispensable for activation of the late promoters. Loss of Carm1 results in a loss of Brg1 association with myogenic promoter elements indicating that Carm1/Prmt4 binding at late- myogenic promoters facilitates binding of the Brg1 ATP-dependent chromatin-remodeling enzyme and subsequent chromatin remodeling at these regulatory sequences.

These studies collectively demonstrate that epigenetic regulators impact almost every step of myogenesis. DNA methylation by Dnmts regulates skeletal muscle specific MyoD expression, commitment to the myogenic lineage requires Pax7-mediated recruitment of Wdr5–Ash2L–MLL2 that upregulates Myf5 expression, control of cell cycle exit and S-phase genes is mediated by HDAC1, Suv39h1; MLL5 plays a role in quiescence and maintenance of determination gene expression; and MyoD and MEF2 activities during differentiation are controlled through complexes of HDACs, HMTs, HATs and the SWI/SNF remodeling enzymes.

7.6 Targeting Epigenetic Regulators in Muscular Dystrophy

Given the impact of HDACs on inhibition of MyoD and MEF2 activities, treatment of myoblasts with HDAC inhibitors was found to result in increased size of muscles. Interestingly, the inhibition of HDACs resulted in the upregulation

of a key target gene, follistatin. Follistatin has no impact on proliferation of myoblasts, but instead mediates increased fusion, resulting in hypernucleated myotubes (Iezzi et al. 2004). Follistatin is also a negative regulator of myostatin/TGFβ signaling which inhibits muscle growth and regeneration. Thus, treatment with various HDAC inhibitors such as trichostatin A (TSA), valproic acid, and phenylbuytrate was found to enhance differentiation of satellite cells. Moreover, intraperitoneal injections with HDAC inhibitors resulted in increased follistatin expression and improved muscle fiber size that exhibit increased resistance to degeneration in mdx mice, a mouse model of muscular dystrophy (Minetti et al. 2006). At the molecular level, these findings reflect a regulatory connection of HDAC2 and follistatin expression. HDAC2 is S-nitrosylated by nitric oxide (NO) and this modification blocks HDAC2-mediated repression of follistatin. In DMD, the loss of the dystrophin results in loss of nitric oxide synthase (nNOS), and thus deregulated NO signaling. Consequently, increased HDAC2 activity leads to constitutive repression of follistatin that is blocked with HDAC inhibitors.

Intriguingly, in mdx hearts, an increase in PCAF and p300 expression, but not that of HDAC1 was reported recently (Colussi et al. 2011). This correlated with increase in N^{ε}-Lysine acetylation of connexin 43 and altered localization. Consistently, treatment with a HAT inhibitor anacardic acid, restored connexin 43 localization and cardiomyopathy in mdx mice. Moreover, the activity of HDAC4 was reduced in dystrophic heart, indicating that the levels of PCAF and HDAC4 may be important in connexin 43 localization. Thus, while HDAC inhibitors may present a viable pharmacological option for therapeutic intervention in muscular dystrophies, the consequences and impact of global HDAC inhibition through systemic treatments with HDAC inhibitors needs further consideration. Contemplation of tissue specific effects is essential in the use of drugs targeting epigenetic regulators for myopathies where systemic delivery is required.

7.7 Conclusion

In this review we have attempted to summarize some of the mechanisms which regulate the commitment and differentiation of cells into the myogenic lineage, and the role of epigenetics in developmental and adult skeletal myogenesis. Many chromatin modifiers and remodeling enzymes play both positive and negative roles at distinct steps to tightly control the initiation and progession of the differentiation program activity. In addition, recent studies have documented striking advances in the possibility of using epigenetic regulators in muscle pathologies. In light of these exciting developments, it is clear that further understanding of various epigenetic modifiers will facilitate and accelerate potential therapeutic strategies in myopathies that result from altered proliferation and differentiation of muscle precursor cells, and continue to push forward discoveries in this important area of research.

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Chapter 8
Small Changes, Big Effects: Chromatin Goes Aging

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Abstract Aging is a complex trait and is influenced by multiple factors that are both intrinsic and extrinsic to the organism (Kirkwood et al. 2000; Knight 2000). Efforts to understanding the mechanisms that extend or shorten lifespan have been made since the early twentieth century. Aging is characteristically associated with a progressive decline in the overall fitness of the organism. Several studies have provided valuable information about the molecular events that accompany this process and include accumulation of nuclear and mitochondrial mutations, shortened and dysfunctional telomeres, oxidative damage of protein/DNA, senescence and apoptosis (Muller 2009). Clinical studies and work on model organisms have shown that there is an increased susceptibility to conditions such as neurological disorders, diabetes, cardiovascular diseases, degenerative syndromes and even cancers, with age (Arvanitakis et al. 2006; Lee and Kim 2006; Rodriguez and Fraga 2010).

Investigations into aging mechanisms in unicellular systems, like yeast and *in vitro* cell culture models, have identified several pathways involved in this process. In cells aging is typically associated with a senescent phenotype. Cells are known to have a limited proliferative capacity (Hayflick limit) (Hayflick 1965) and senescence can be defined as a state in which cells cease to proliferate after a finite number of divisions (Adams 2009). Some of the well-known triggers that induce senescence include DNA damage, telomere shortening and redox stress (Rodier and Campisi 2011). From literature, it is evident that in most of these cases, factors/pathways which bring about cell cycle arrest are activated and include p53/p21, and p16/RB

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pathways (Ben-Porath and Weinberg 2005). However, the cellular/molecular signatures that characterize senescence are typically scored by induction of senescence associated β-galactosidase activity, marks of cell cycle arrest, changes in cellular morphology and/or organization, secretion of numerous proteins including cytokines and chemokines, and DNA damage (Rodier and Campisi 2011). The quest to decipher the molecular events that induce or bring about cellular senescence have unraveled the role of chromatin as an important component of this response (Misteli 2010).

The DNA in every eukaryotic cell exists as a complex with specialized proteins called histones that form chromatin. Chromatin plays a central role in processes that range from gene expression to chromosome dynamics during the cell cycle. Chromatin can be broadly categorized into two types, namely, euchromatin and heterochromatin (Bassett et al. 2009). Euchromatin appears decondensed cytologically and is mostly transcriptionally active. Heterochromatin, on the other hand, is highly compact and mostly contains transcriptionally silenced genes (Bassett et al. 2009; Frenster et al. 1963). The building block of chromatin is a nucleosome that consists of 147 base pairs of DNA wrapped around a protein octamer containing two molecules of each canonical histone H2A, H2B, H3 and H4, and is separated from one another by 10–60 base pairs of linker DNA (Luger et al. 1997). Histones contain a typical histone-fold domain, which is required to form the octamer, and their N-termini protrude out of the nucleosomes (Luger et al. 1997). Most of the residues on these histone tails are subject to posttranslational modifications (Jenuwein and Allis 2001). Some of the most prevalent modifications of histones are phosphorylation, acetylation, methylation, ubiquitination, sumoylation, ADP-ribosylation and biotinylation (Jenuwein and Allis 2001; Margueron et al. 2005). Recent reviews have illustrated biophysical and physiological consequences of such modifications on chromatin structure and function (Li and Reinberg 2011). In addition to histone and non-histone proteins that bind to DNA, modification of DNA (Cytosine methylation in eukaryotes) is also an important component of chromatin (Li and Reinberg 2011). It is interesting to note that there is a dynamic interplay between histone and DNA modifications that determine chromatin structure/function (Bonasio et al. 2010; Li and Reinberg 2011).

As mentioned earlier, the most obvious associations of DNA with aging are increased DNA damage (or reduced repair) (Seviour and Lin 2010) and telomere shortening (Kenyon and Gerson 2007). Increasing evidence in literature indicates that chromatin plays a major role in affecting both these processes (Shin et al. 2011a). In addition to these, the ability of chromatin to affect gene expression patterns in a cell has huge consequences on the ability to maintain homeostasis. Therefore, given the central role of chromatin in affecting various cellular processes, intuitively one would expect it to be a crucial component of cellular aging. In this chapter, we review the recent progress on the role of chromatin in aging. Specifically, we highlight the chromatin changes that have been associated with cellular and/or organismal aging. Importantly, we also highlight the role of histone modifiers in affecting lifespan.

8.1 Chromatin and Aging

8.1.1 DNA Methylation

DNA methylation, one of the most well studied epigenetic marks, involves the methylation of cytosines in CpG dinucleotides and is catalyzed by enzymes termed as DNA methyl transferases (DNMTs: DNMT1, DNMT3a, DNMT3b) (Jurkowska et al. 2011). It is well established that DNA methylation constitutes mechanisms required for both short-term and long-term effects on gene expression (Bonasio et al. 2010; Li and Reinberg 2011). Specifically, alterations in methylation of CpGs at upstream regulatory elements are known to modulate transcription of genes (Li and Reinberg 2011). Due to its ability to control both global and locus specific chromatin functions, DNA methylation is known to play a critical role in cellular physiology. It is important to note that key biological processes such as development, differentiation and cell death are affected by DNA methylation (De Carvalho et al. 2010; Geiman and Muegge 2010; Gibney and Nolan 2010). Its role in aging and/or senescence has been addressed in the recent past, and it is apparent that DNA methylation is one of the key factors involved in cellular and/or organismal aging (Feser and Tyler 2011; Fraga and Esteller 2007; Sedivy et al. 2008; Dimauro and David 2009). Figure 8.1 illustrates the changes in DNA methylation during aging.

An important role for DNA methylation in aging was first evidenced in replicative senescence of primary fibroblasts from mice, hamsters and humans. The study showed that in these cells, levels of 5-methylcytosine markedly declined during senescence (Wilson and Jones 1983). A follow up study demonstrated that accelerated 5-methylcytosine loss (by 5-azacytidine treatment) shortened the *in vitro* lifespan of human diploid fibroblasts (Fairweather et al. 1987). This phenomenon was reconfirmed by various *in vivo* and *in vitro* studies wherein it was observed that DNA methylation levels fell during aging, both at certain specific loci and at a genome wide level (Fairweather et al. 1987; Christensen et al. 2009; Fuke et al. 2004; Singhal et al. 1987). Interestingly, recent reports have indicated that this age-associated decline in total genomic DNA methylation occurs mostly at repetitive DNA sequences (Koch et al. 2011; Romanov and Vanyushin 1981; Singhal et al. 1987; Wilson et al. 1987). These observations have led to the speculation that the decrease in DNA methylation affects constitutive heterochromatin (DePinho 2000). Specifically, it has been suggested that with age de-heterochromatinization of repetitive regions could lead to deleterious recombinations which may cause increased incidences of age-associated diseases such as cancer (DePinho 2000). Further, the importance of DNA methylation in aging is supported by observations that show age-dependent decrease in the expression of the DNA methyltransferase (DNMT1) (see below) (Casillas et al. 2003; Lopatina et al. 2002). Supporting that the gradual loss of DNA methylation could function as a "counting hypothesis" for senescence (Hoal-van Helden and van Helden 1989; Wilson and Jones 1983), CpG methylation was shown to decrease with increased population doublings of normal cells in

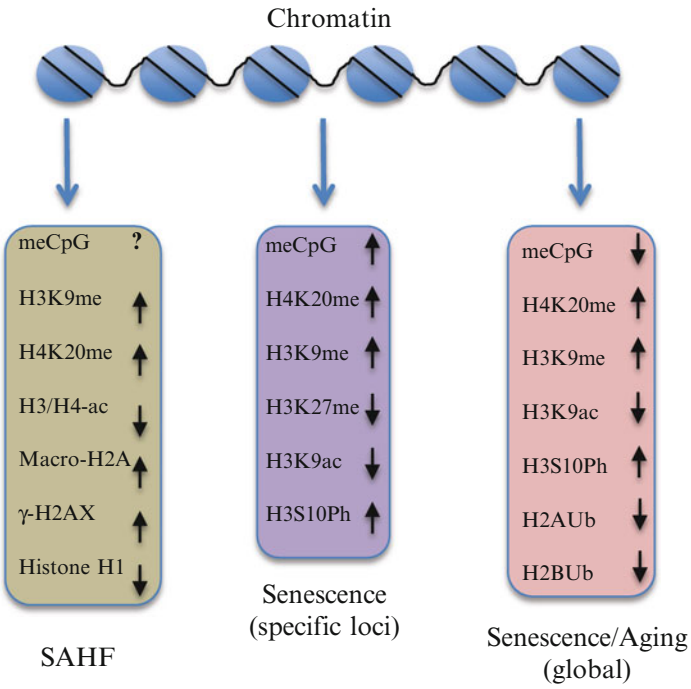

Fig. 8.1 Epigenetic changes during aging. Alterations in epigenetic marks in cultured cells and/or tissues during aging affects chromatin both globally and at specific loci. In general, histone modifications associated with heterochromatin seem to accumulate during aging. The association between DNA methylation and aging is context dependent and often determined by global and locus specific changes

culture (Fairweather et al. 1987; Wilson and Jones 1983) and during organismal aging (Hornsby et al. 1992; Singhal et al. 1987).

Contrary to observations of a decrease in global DNA methylation during aging specific loci and promoter regions of key cell cycle regulatory genes have been shown to be hypermethylated (Fig. 8.1). For example, the Estrogen receptor gene (Issa et al. 1994), *INK4A/ARF/INK4b* locus (which codes for p16, p14 and p15 proteins respectively) (Koch et al. 2011), ribosomal RNA genes (Swisshelm et al. 1990; Oakes et al. 2003) and multiple tumor suppressor or tumour-associated genes like APC and E-cadherin (Bornman et al. 2001; Waki et al. 2003) accumulate DNA methylation during aging. Interestingly, in another study it was observed that DNA methylation levels were maintained in long-term culture of mesenchymal stromal cells (MSE) and MSEs from young and old donors. However, they exhibited differential DNA methylation patterns at specific loci, like in the homeobox genes and genes involved in cell differentiation (Bork et al. 2010). It has been hypothesized that this locus specific hypermethylation in the background of a global reduction of

methyl-CpGs could be due to an increase in Dnmt3b expression that has been observed in senescent cells (Casillas et al. 2003; So et al. 2006).

Observations from some studies suggest that hypermethylation depends upon the prevalent density of methyl-cytosines at specific loci with sparsely methylated regions more amenable to hypermethylation (Song et al. 2002; Stirzaker et al. 2004). These observations have led to a 'seeds of methylation' hypothesis based on increasing CpG methylation levels (Rakyan et al. 2010). A recent genome-scale study addressed dynamic changes in the epigenome in normal human aging (Rakyan et al. 2010). This report identified aging-associated differentially methylated regions (aDMRs) that gain methylation with age in different tissues, thus suggesting that aDMR signature is a multi-tissue phenomenon. Further, it was also demonstrated that aging associated DNA hypermethylation occurs predominantly at bivalent chromatin/promoters (Rakyan et al. 2010). These studies point out an interesting aspect of locus specific methylation contributing to aging. In this scenario one would expect an inherent bias in methylation rates at loci that would ultimately (or cumulatively) result in a senescent phenotype.

8.1.2 Histone Modifications

As mentioned in the introduction, histone modifications are one of the most central elements that affect chromatin structure and function. The most common and well-studied histone modifications that are known to impact chromatin are acetylation of lysines, methylation of lysines and arginines, phosphorylation of serine and threonine, and ubiquitination of lysines (Jenuwein and Allis 2001; Margueron et al. 2005). It is evident from literature that interfering with these modifications affects both global and locus specific chromatin, and as a consequence impinges on various cellular processes (Murr 2010). Some recent reviews provide exhaustive information about histone modifications and their role in chromatin structure and function. Figure 8.1 illustrates the histone modifications, which are associated with 'open or closed' chromatin and their associations with aging.

Specific histone modifications undergo distinct changes in profile during aging (Fig. 8.1). The levels of histone H4 lysine-20 tri-methylation (H4K20Me3), a mark of constitutive heterochromatin and which is enriched in differentiated cells, have been found to increase in senescent cells. This has been speculated to cause the accumulation of heterochromatic structures in senescent human fibroblasts (Narita et al. 2003). The total abundance of histone H4K20Me3 has also been reported to increase with age in rat liver and kidney (Kouzarides 2007; Sarg et al. 2002), supporting the notion that heterochromatin may accumulate with tissue aging, at least at some sites. In another study, Bracken et al. observed a loss of histone H3 lysine-27 tri-methylation (H3K27Me3), a mark associated with silent chromatin, at the *INK4b* and *INK4a–ARF* loci in senescent human diploid lung embryonic fibroblast cell line (Bracken et al. 2007). This decrease in H3K27Me3 was accompanied by a decrease in EZH2, the histone methyltransferase responsible for this modification

(Bracken et al. 2007). Several groups have studied changes in histone H3 modifications with age in rat liver. They found that histone H3 lysine-9 acetylation (H3K9Ac) decreased and histone H3 Serine-10 phosphorylation (H3S10Ph) increased with age significantly (Braig et al. 2005; O'Sullivan et al. 2010; Kawakami et al. 2009). These independent observations both in cells in culture and in aged animals clearly establish a positive correlation between heterochromatic marks and aging (Fig. 8.1).

Mono-ubiquitination of histones H2A and H2B is known to alter chromatin dynamics and regulate gene expression. While H2A ubiquitination leads to silencing, ubiquitination of H2B has been implicated in active transcription. Interestingly, these modifications have been associated with aging. The link between histone ubiquitination and aging was first demonstrated by a study which showed that the proportion of ubiquitinated histones was about 30% higher in old mice than in young ones (Morimoto et al. 1993). However, reduced expressions of H2B ubiquitin ligases RNF20/Bre1 have been associated with senescence/aging phenotypes. In yeast, absence of Bre1 results in reduced lifespan during chronological aging due to enhanced apoptotic cell death (Walter et al. 2010). Similarly, depletion of RNF20 has been shown to induce cellular senescence in glioma cells (Gao et al. 2011). Like H2B, ubiquitination of histone H2A has also been implicated in aging. Downregulation of BMI1, a component of the polycomb repressive complex (PRC), which ubiquitinates histone H2A (Cao et al. 2005) has been shown to result in derepression of growth inhibitory genes and putative tumor suppressors. As a consequence these cells display premature senescence and apoptosis (Bommi et al. 2010). Although, these studies suggest that histone ubiquitination is involved in aging, whether these effects are mediated through alterations in global chromatin architecture or transcription of specific genes is still not clear.

8.1.3 Senescence Associated Heterochromatic Foci (SAHF)

Cells grown in culture have provided valuable insights into aging mechanisms. In this regard, most of our understanding of the role of chromatin on aging has come from studies on senescing cells. Not surprisingly, alterations of chromatin structure are associated with the irreversible state of senescent cells (Braig and Schmitt 2006; Narita et al. 2003). Many senescent human cells, when stained with the DNA staining dye 4′, 6-diamidino-2-phenylindole (DAPI), show visible punctuate DNA foci known as senescence associated heterochromatic foci (SAHF), a new type of facultative heterochromatin (Narita 2007; Narita et al. 2003). RNA-FISH and *in situ* labeling of nascent RNAs demonstrate that SAHF contain transcriptionally inactive chromatin (Funayama et al. 2006; Narita 2007). In addition to being transcriptionally inactive, these loci are also enriched with typical proteins that are found in heterochromatin. For example, SAHFs in general contain heterochromatin protein-1 (HP1), repressive histone modifications like H3K9 methylation and hypoacetylated histones (Narita et al. 2006). However, these SAHFs do not show some usual marks of condensed chromatin, like the phosphorylation of histone H3 at Serine-10 or

Serine-28, marks of mitotic chromatin or of histone H2B at Serine-14, a mark of apoptotic chromatin (Funayama et al. 2006; Peterson and Laniel 2004).

It is important to note that in addition to changes in histone modifications, histone chaperones, and alterations in chromatin composition have also been implicated in senescence. For example, studies have shown that the formation of SAHF during cellular senescence depends on histone H3 chaperones, ASF1 (anti-silencing function 1) (Zhang et al. 2005) and HIRA (histone cell cycle regulation defective homologue A) (Ye et al. 2007). Interestingly, these loci are also known to contain variants of histones which have been otherwise associated with silenced chromatin. SAHFs are enriched with macro-H2A (histone H2A variant) that is mainly required for inactivation of X-chromosome (Costanzi and Pehrson 1998; Funayama et al. 2006; Zhang et al. 2005). The role of histone variants in the formation of SAHF is also supported by findings that report an increase in γ-H2AX in early neoplastic lesions that contain senescent cells *in vivo* and also in aging tissues. This is thought to contribute to senescence and proliferation arrest of damaged cells (Bartkova et al. 2006; Herbig et al. 2006). Chromatin compaction can also be altered by the recruitment of factors that are known to replace histone HI and bind to linker DNA. In this regard, the finding which shows that in SAHFs there is a decrease in linker histone H1 occupancy and increased levels of chromatin-bound high mobility group-A proteins (HMGA) becomes relevant (Funayama et al. 2006; Narita et al. 2006). The exact molecular mechanisms of SAHF formation are not very clear, but independent studies have demonstrated that the ectopic expression of either HMGA1 or HMGA2 induces SAHF formation and other senescence phenotypes in normal human fibroblasts. It was also observed that knockdown of HMGA proteins by RNAi prevents SAHF formation, thus indicating that HMGA are essential components for SAHF formation (Funayama et al. 2006; Narita et al. 2006). It has been speculated that the DNA-bending properties of HMG family proteins may help induce SAHF formation by binding and bending linker DNA (Hock et al. 2007; Paull et al. 1993).

Formation of heterochromatin is often facilitated by enzymatic activities that are known to repress transcription. Notably, histone deacetylases and histone methyl transferases that add 'repressive chromatin marks' play essential roles in heterochromatin formation. The Sin3 multiprotein complex is a repressor complex recruited by several sequence specific transcription factors. The repressor activity of the Sin3 complex is brought about by the Sin3A/Sin3B-associated HDAC1 and HDAC2 proteins. A study by Grandinetti et al. has demonstrated that Sin3B-null fibroblasts are resistant to replicative and oncogene-induced senescence (Grandinetti et al. 2009). They also showed that over-expression of Sin3B triggers senescence and the formation of SAHF. However, the role of histone deacetylation in inducing SAHF seems to be HDAC specific. While Sin3 complex via HDAC activity aids in the formation of SAHFs, a study by Huang et al. has suggested that Sirt1, a NAD^+-dependent deacetylase (described below), antagonizes cellular senescence in human diploid fibroblasts (Huang et al. 2008). Their experiments demonstrated that over-expressing Sirt1 led to a reduction of senescence associated biomarkers, which included the formation of SAHFs (Huang et al. 2008).

Although, SAHFs seem to bring about a global change in chromatin architecture, it is not clear if SAHF formation contributes to senescence. In support of SAHF contributing to senescence, evidence show that SAHF formation contributes to stable proliferative arrest by repressing transcription of E2F target genes that are required for G1 to S phase transition (Narita et al. 2003). Chromatin immunoprecipitation analyses have demonstrated that the promoters of E2F target genes become heterochromatic in senescent cells but not in proliferating or quiescent cells. In addition, overexpression of E2F-1 was not able to derepress these genes indicating heterochromatinization mediated transcriptional silencing (Narita et al. 2003). Interestingly, SAHF-dependent silencing of E2F genes requires the retinoblastoma (Rb) protein at these gene promoters (Narita et al. 2003). Further, studies have shown that Rb associates with HP1 and the histone methyltransferase Suv39H1 to facilitate senescence. Specifically, Rb family members have been shown to interact with HDAC1, DNA methyltransferase and polycomb proteins among other transcriptional co-repressors to repress the activity of E2F1 (Trimarchi and Lees 2002; Narita et al. 2003). Prohibitin, a protein implicated in cell cycle control and antiproliferative activities, is found in SAHF and colocalizes with HP1 (Rastogi et al. 2006). This finding suggests that SAHFs might actively contribute to senescence. In this study prohibitin, Suv39H1 and HP1 were detected on E2F target promoters during senescence, and a deletion of prohibitin led to a loss of senescent phenotype (Rastogi et al. 2006).

Although, there is a lot of evidence to suggest that SAHF formation is important for induction of senescence, formation of heterochromatin itself seems to be the most important feature of senescence. A recent study has shown an increase in the abundance of heterochromatin proteins and marks in senescence but without the formation of SAHF (Kosar et al. 2011). Hence, local heterochromatinization, but not global SAHF, may induce senescence-associated proliferation arrest by mediating the silencing of proliferation genes.

8.1.4 microRNAs, Epigenetics and Aging

MicroRNAs are ~22 bases long RNAs, which bind to the 3′UTR of target mRNAs and regulate gene expression post-transcriptionally by translational inhibition or mRNA degradation (He and Hannon 2004). Due to their ability to target multiple mRNAs, they are now considered as major factors that affect cellular physiology (He and Hannon 2004; Sayed and Abdellatif 2011). Originally appreciated for their role in cancer and development, microRNAs have also been shown to be involved in regulating factors or pathways, which impinge on aging. In the recent past, studies have identified many microRNAs that target ‘aging’ factors and several reviews have highlighted these reports (Bates et al. 2009; Gorospe and Abdelmohsen 2011; Grillari and Grillari-Voglauer 2010). Rather than detailing microRNAs and their targets that have been implicated in aging, we specifically highlight studies that have addressed altered expression of microRNAs during aging.

Intriguingly, global microarray profiling studies suggest that more microRNAs are upregulated rather than downregulated during aging (Li et al. 2011; Maes et al. 2008; Zhang et al. 2010). It is important to note that upregulation of some of these microRNAs have been implicated in regulating the expression of genes, which are known to affect organismal physiology. For example, miR-669c and miR-709 (up-regulated at 18 months with a maximum expression at 33 months), and miR-93 and miR-214 (up-regulated around 33 months) have been shown to target genes associated with detoxification and regenerative capacity of the liver, functions that slowly decline in aged liver (Maes et al. 2008). In another study, Bates et al. profiled microRNAs regulated in the liver of Ames dwarf mice, which display a delayed onset of aging (Steuerwald et al. 2010). They found that miR-27a is upregulated in these dwarf mice at an early age. Their results also suggest that miR-27a regulates two key metabolic proteins ornithine decarboxylase and spermidine synthase. Based on these observations the authors have speculated that miR-dependent regulation of metabolic pathways such as glutathione metabolism, urea cycle, and polyamine biosynthesis maybe important for health span and longevity in these mice (Bates et al. 2010). However, studies which link microRNAs with DNA repair or cell proliferation pathways have raised the possibility that age related alterations in microRNA expressions maybe relevant in mediating the aging process (Chen et al. 2010).

The link between microRNAs and aging has been further strengthened by a study in which reducing the activity of *C. elegans* linage 4 (*lin-4*) microRNA shortened lifespan and its overexpression led to a longevity phenotype (Boehm and Slack 2005). Another study that looked at senescence in normal human keratinocytes (NHK) found microRNAs miR-137 and miR-668 to be upregulated during replicative senescence (Shin et al. 2011b). Interestingly, induction of senescence by ectopic over-expression of miR-137 and miR-668 was associated with an increase in senescence associated (SA) β-galactosidase activity, p53 and p16INK4A levels. Further, expressions of these microRNAs were also observed to be elevated during organismal aging of normal human oral epithelia (Shin et al. 2011b).

Although, it is increasingly becoming apparent that microRNAs play a vital role in regulating aging, very little is known about epigenetic changes that mediate the expression of such key microRNAs. Recent studies have clearly shown that microRNA expression is regulated by epigenetic marks (Liang et al. 2009). Importantly, their promoters have been shown to exhibit differential DNA methylation and histone modifications, that are reminiscent of modifications on protein coding genes (Lee et al. 2011; Saito and Jones 2006). Lee et al. have demonstrated that inhibition of HDACs triggers cellular senescence by inducing the expression of miR-23a, miR-26a and miR-30a. Interestingly, these microRNAs target and downregulate HMGA2 expression that has been associated with induction of senescence (Lee et al. 2011).

Further studies aimed at profiling microRNAs during aging, and in specific tissues, will aid in appreciating the regulation of pathways that mediate lifespans of organisms. Importantly, investigating the mechanisms that control mircoRNA expression, specifically histone deacetylases and DNA methyltransferases

(which have been associated with aging, see below), will highlight the importance of posttranscriptional control of 'aging genes'. In addition, such insights will provide a holistic picture of changes in gene regulation, mediated by chromatin modifiers, in affecting organismal longevity.

8.2 Role of Chromatin Modifiers in Aging

The previous section highlights the importance of chromatin associated changes in aging and cellular senescence. Although, it is clear that these changes are strong correlates of aging, whether they are causal factors or mere consequences of aging remains unclear (Dimauro and David 2009). Also, aging/senescence dependent changes that the enzymes which affect these modifications themselves undergo are less appreciated. As reviewed elsewhere, post-translational modifications of histones are catalyzed by specific enzymatic machineries (Bannister and Kouzarides 2011). Histone acetylation is affected by opposing activities of histone acetyltransferases (HATs) and histone deacetylases (HDACs) (Legube and Trouche 2003). Separate families of enzymes are known to methylate and demethylate lysine/arginine residues in histones (Yoshimi and Kurokawa 2011). Interestingly, unlike these modifications histone phosphorylation and dephosphorylation are brought about by a diverse set of enzymes (Hans and Dimitrov 2001). In this section, we have attempted to review the studies which have given us insights into the role of histone modifiers during aging. Specifically, we will look at the important classes of chromatin modifiers: DNMTs, Histone acetyltransferases Histone deacetylases and Sirtuins (Table 8.1).

Table 8.1 List of chromatin modifiers and their association with aging or senescence

Chromatin modifier	Modification site	Modification	Alteration with age	Role in aging
DNMT1	CpG dinucleotide	Methylation	Decrease	Anti-senescent
DNMT3a	CpG dinucleotide	Methylation	Increase	Locus specific effects
DNMT3b	CpG dinucleotide	Methylation	Increase	Locus specific effects
Mof (HAT)	H4K16	Acetylation	Increase	Pro-senescent
Sas2 (HAT)	H4K16	Acetylation	Increase	Pro-senescent
CBP (HAT)	H3K9, H3K27, H3K56	Acetylation	Decrease	Anti-senescent
P300 (HAT)	H3K9, H3K27, H3K56	Acetylation	Decrease	Anti-senescent
HDAC1	H3K9, H3K56, H4K16	Deacetylation	Increase	Pro-senescent
SIRT1	H3K9, H4K16	Deacetylation	Decrease	Anti-senescent
SIRT6	H3K9	Deacetylation	?	Anti-senescent
EZH2 (HMT)	H3K27	Methylation	Decrease	Pro-senescence

The table illustrates the roles of DNMTs, HMTases, HATs, HDACs and Sirtuins, and depicts changes in their expression during aging

8.2.1 DNA Methyl Transferases (DNMTs)

DNA methyltransferases (DNMTs) catalyze the addition of a methyl group to DNA on cytosines, typically in CpG dinucleotides. DNA methylation has been associated with gene silencing and robust regulation of transcription. DNMT mediated DNA methylation brings about chromatin silencing by inducing the formation of heterochromatin through recruitment of specific proteins like Methyl CpG binding proteins, MeCP2 (Kimura and Shiota 2003) and MBD (Fujita et al. 2003; Villa et al. 2006). It is interesting to note that DNMTs have been shown to be in complex with histone methyl transferases and histone deacetylases. In mammals there are three DNA methyltransferases, namely, Dnmt1, Dnmt3a and Dnmt3b. Dnmt1 is considered as a maintenance methylase since it methylates newly replicated DNA using hemimethylated DNA as a substrate. Dnmt3a and 3b mediate *de novo* methylation, that is, they can methylate previously unmethylated DNA.

The role of DNMTs in aging has been addressed in the recent past because of their ability to affect chromatin/epigenetic modifications. In addition, previous reports have also correlated changes in DNA methylation during aging. Several studies have thrown light upon changes in DNMT levels and activity that may have crucial roles in cellular senescence (Lopatina et al. 2003; Vogt et al. 1998). Consistent with previous observations of a decrease in global DNA methylation in senescence, studies have shown that the levels and activity of the maintenance methylase Dnmt1 decrease in aging fibroblast cells. However, an increase in Dnmt-3a and -3b activity was observed which raises the possibility of a compensatory role for these DNMTs (Casillas et al. 2003; Lopatina et al. 2003). Although, it was long observed that promoter hypermethylation of cell cycle inhibitory genes, $p16^{INK4A}$ and $p21^{CIP1/WAF1}$ tipped the balance between senescence and oncogenesis, a recent report shows the involvement of DNMTs, and their findings have provided further support to the hypothesis that DNMTs are important for inducing senescence. The authors of this study observed that upon inhibition of DNMT1 and DNMT3b in human umbilical cord blood-derived multipotent stem cells (hUCB-MSCs), p16 and p21 expression increased and activated senescence in these cells (So et al. 2011). Not surprisingly, several studies have shown that DNMTs are overexpressed in cancer cell lines resulting in silencing of the expression of p16 (So et al. 2011; Yang et al. 2001).

Calorie/Dietary restriction (CR/DR) is one of the interventions that has been commonly used to understand the molecular factors involved in aging. Although, as previously mentioned it is unclear whether DNMTs play a deterministic role in aging, studies have indicated that Dnmt3a levels in the mouse hippocampus change when the animals are subjected to dietary restriction (Chouliaras et al. 2011a, b). This study also sheds light on the possibility that one of the major mechanisms by which CR/DR mediates organismal aging is by modulating DNA methylation status by regulating expression levels/enzymatic activities of individual DNMTs. However, it is still unclear if DNMTs can by themselves induce senescence or whether other factors that initiate or require chromatin changes affect their expression and/or activities during aging. Nevertheless, these findings clearly indicate that the changes

in DNMT expressions can lead to alterations in chromatin structure during aging by influencing both global and gene specific methyl-CpG levels and/or distribution. In spite of these reports that indicate strong associations between DNMT expression/activities, DNA methylation patterns and aging, it is surprising to find that there are very few attempts to map the changes in DNMT localizations on a genome wide scale. Further, it would be interesting to similarly analyze genome-wide alterations in DNA methylation profiles in various model systems that are known to extend their lifespans in response to dietary interventions.

8.2.2 Histone Acetyl Transferases and Histone Deacetylases

Besides DNA methylation, histone modifications have become the most important determinants of chromatin structure/function involved in various cellular outputs. Specifically, histone acetylation has been one of the hallmarks of active gene transcription and often influences other modifications of histones such as methylation. As previously mentioned, histone acetylation-deacetylation reactions are catalyzed by histone acetyl transferases and histone deacetylases, respectively. These enzymes have been implicated in aging from studies that have attempted to decipher the changes observed in histone modifications during aging and/or map the genetic factors involved in aging. Although, very little is known about the role of HATs, HDACs have been well addressed with regards to their involvement in cellular or organismal aging.

HATs: An important HAT that has been linked to aging is Mof, which mediates acetylation at the H4 lysine 16 (H4K16) residue. Mof has been shown to be important for the maintenance of genome stability and its depletion leads to delayed γ-H2AX foci formation in response to DNA damage and abrogated DNA damage repair. Mof has also been shown to be an important regulator of DNA damage because of its ability to bind to 53BP1 (Krishnan et al. 2011). Mof associates with the nuclear matrix and is a key component of the pre-lamin A complex. In a very recent study, Vaidehi Krishnan et al. have shown that in mice that lack the zinc metalloproteinase (Zmpste 24), Mof localization at the nuclear matrix decreases (Krishnan et al. 2011). This effect has been linked to the accumulation of unprocessed pre-lamin A, which is associated with progeroid symptoms. Incidentally, depletion of Mof has also been shown to exacerbate the senescent phenotype of cell lines that lack Zmpste 24. In support of this, overexpression of Mof has been associated with hyperacetylation of H4K16 and a delay in cellular senescence (Hajji et al. 2010). Another HAT that seems to be an important mediator of aging is Sas2. Studies in *S. cerevisiae* have shown that Sas2 inactivation leads to delayed senescence due to activation of homologous recombination (HR) machinery at telomeric regions, thus delaying senescence by preventing telomere loss (Kozak et al. 2010).

Several studies show that the HATs p300 and CBP are important regulators of senescence (Bandyopadhyay et al. 2002; He et al. 2011; Pedeux et al. 2005; Prieur

et al. 2011). The study by Prieur et al. showed that p300 is an important regulator of chromatin dependent mediator of senescence and that this mechanism is independent of p53, p21 and p16 (Prieur et al. 2011).

HDACs: There are four classes of HDACs and specifically, Sirtuins that belong to Class-III HDACs are distinct in their activity because of their dependence on NAD^+. We have described the role of sirtuins in aging in a separate section below. Among the other HDACs, members that belong to Class I have been so far implicated for their potential roles in aging. Several studies have indicated that inhibition of HDAC activity leads to induction of a senescent phenotype. June Munro et al. in their study show that administration of HDAC inhibitors sodium butyrate and trichostatin A (TSA) induces senescence in human fibroblasts. These cells exhibit typical senescent phenotype such as β-galactosidase staining, in addition to an elevation in cyclin-Cdk inhibitors, p21and p16 (Munro et al. 2004). It is interesting to note that HDAC antagonists are potent inhibitors of cancer cell proliferation or tumorigenesis. Suberoylanilide hydroxamic acid (SAHA), is an HDAC inhibitor which is used as an anti-tumor drug. SAHA has been shown to induce polyploidy in human colon cancer cell line HCT116 and human breast cancer cell lines, MCF-7, MDA-MB- 231, and MBA-MD-468, which activates senescence in these cells (Xu et al. 2005). Results that corroborate these findings also show that senescence is accompanied by a decrease in HDAC expression. Contradictory observations regarding the role of HDAC activity and aging have also been made. A recent study showed that HDAC1 overexpression inhibited cell proliferation and induced premature senescence in cervical cancer cells through a pathway that involved the deacetylase Sp1, protein phosphatase A PP2A and retinoblastoma protein Rb (Chuang and Hung 2011).

Contrary to what has been observed in cells in culture, organismal studies have indicated that a reduced/absence of HDAC expression/activity leads to lifespan extension. Rpd3 is an HDAC found in all organisms. A study by Rogina et al. showed that reduction in Rpd3 levels in *Drosophila* renders them a longer lifespan. Moreover, these mutants fail to increase their lifespan any further in response to CR/DR (Rogina and Helfand 2004). It has to be noted that the molecular mechanisms of HDAC-dependent changes in aging and lifespan are not well understood. Interestingly, administering TSA to flies also leads to an extension in lifespan, which is accompanied by an increase in Hsp22 protein levels (Tao et al. 2004; Zhao et al. 2005). However, the global changes in histone acetylation and chromatin architecture that would be associated with an absence or inhibition of HDACs have not been addressed, yet. It is unclear if these results depict physiological differences which are elicited by HDAC inhibition at the cellular and organismal levels. It is also likely that different family members which belong to HDACs have varied roles and involve altered substrate specificities. It has to be noted that HDACs are known to deacetylate and affect non-histone proteins as well (Thevenet et al. 2004; Gregoire et al. 2007). Further analysis of individual HDAC proteins may identify their individual functions in mechanisms that induce senescence.

8.2.3 Sirtuins

Sir2 is the founding member of an evolutionarily conserved family of proteins that was first identified in *S. cerevisiae*. Sir2 has been classified as a Class III HDAC and it has been shown to depend on NAD^+ as a co-substrate for its deacetylase activity (Ghosh et al. 2010; Imai and Guarente 2010; Zhang and Kraus 2010). The role of Sir2 in regulation of chromatin has been attributed to its ability to deacetylate specific residues in histones H3 (lysine-9) and H4 (lysine-16). Sir2 activity is required to silence chromatin at sub-telomeric DNA, mating-type and ribosomal DNA (rDNA) loci (Fig. 8.2) (Ha and Huh 2011; Kaeberlein et al. 1999; Rusche et al. 2003). Further studies in yeast established Sir2 as a key link between chromatin regulation and aging. In *S. cerevisiae*, it is known that recombination at rDNA regions leads to the formation of extra chromosomal rDNA circles, which reduce replicative lifespan (Sinclair and Guarente 1997). The ability of Sir2 to mediate silencing at rDNA is considered paramount for its role as a negative regulator of aging in yeast. Additionally, a study by Dang et al. shows that in replicatively aged yeast cells, a decline in Sir2 levels correlates with an increase in acetylation of H4K16 (Dang et al. 2009).

Studies aimed at deciphering an "anti-aging" function of Sir2 in worms and flies corroborated the findings in yeast. In *C. elegans*, Sir2 is known to extend lifespan and interact with insulin IGF signaling. Similar reports in *D. melanogaster* have shown that the Sir2 ortholog mediates CR/DR dependent lifespan extension (Rogina and Helfand 2004). However, the molecular mechanisms, which are affected by Sir2 in these organisms that regulate lifespan extensions, are still unclear. The chromatin regulatory function of Sir2 orthologs in other organisms (flies and mammals) has also been addressed. From these studies it becomes evident that the role of Sir2 and its orthologs in regulating chromatin-mediated changes is evolutionarily conserved. Sir2 has been identified as a regulator of heterochromatin formation in flies and affects position effect variegation (PEV). Results indicate that the role of Sir2 in PEV is independent of its ability to extend lifespan (Frankel and Rogina 2005; Newman et al. 2002). However, whether its role in mediating locus specific chromatin changes is linked to its role in lifespan extension is still unclear.

In mammals, SIRT1, the homolog of ySir2 has been shown to be a major regulator of chromatin structure and gene expression. SIRT1 is a well-established regulator of transcription by deacetylating a host of transcription factors and co-regulators (Table 8.2) (Brooks and Gu 2008; Deng 2009). It should be noted that some of these transcription factors, such as NF-kB, FOXO and p53, have been implicated in organismal aging. However, the role of SIRT1 in mammalian aging has been difficult to address as most SIRT1 null mice die due to developmental defects. Mammalian SIRT1 regulates chromatin dynamics by mediating deacetylation of H3 lysine 9 (H3K9) and H4 lysine 16 (H4K16). In addition to contributing to histone acetylation changes, SIRT1 has also been shown to impinge on other mediators of chromatin. Importantly, SIRT1 is known to cross-talk with DNMTs (O'Hagan et al. 2008) and histone methyltransferases (Vaquero et al. 2004). SIRT1 has been shown to bind and

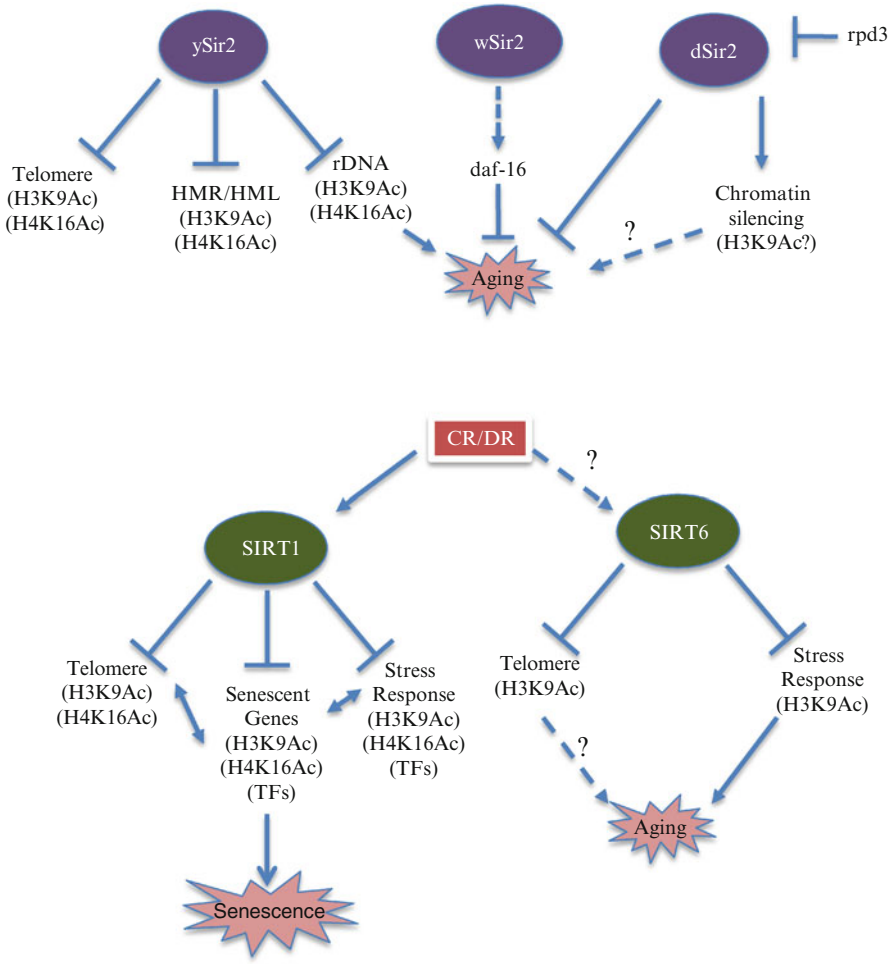

Fig. 8.2 The role of Sirtuins in aging across species. Sir2 and its homologues (including Sirt1 and Sirt6 in mammals) are key players in cellular/organismal aging. Studies in yeast, flies and mammals show that Sir2, Sirt1 and Sirt6 are NAD+-dependent deacetylases and affect chromatin by deacetylating histones (H3K9 or H4K16) as illustrated. Except in yeast, the link between sirtuins and aging is not limited to its role in affecting chromatin since they are known to regulate other pathways/factors. In worms and flies where Sir2 is now known to extend lifespan (and in response to calorie/dietary restriction) the chromatin angle in mediating this effect is still unclear. SIRT1 and SIRT6 are important in regulating the expression of a host of genes that mediate senescence, in addition to their roles at the telomere

deacetylate SUV39H1 which brings about H3K9 trimethylation (Vaquero et al. 2004). It is speculated that SIRT1 mediated histone deacetylation renders the site open for methylation. Additionally, a loss of SIRT1 is associated with a reduction in H3K9me3 levels and a concomitant impairment of heterochromatin protein-1 (HP1)

Table 8.2 Sirt1 deacetylation targets which have been implicated in aging/senescence

Interactor	Biological function
p53	Tumor suppressor and cell cycle regulator
p73	Tumor suppressor and cell cycle regulator
Ezh2	Histone methyl transferase that maintains transcriptionally repressed state
E2F1	A transcription factor important for G1/S transition
PCAF	An acetyltransferase that inhibits apoptosis
RelA/p65	A transcription factor that regulates transcription of NFkB target genes
FOXOs	A family of transcription factors that regulates cell cycle and stress response
SUV39H1	A histone methyl transferase that is important for the maintenance of heterochromatin state

recruitment. Together, these have been proposed to affect heterochromatin formation (Vaquero et al. 2007).

Independent studies have shown that SIRT1 plays a crucial role in cellular senescence. The study by Langley et al. was the first study which showed that SIRT1 negatively regulates cellular aging in mammalian cells (Langley et al. 2002). The authors showed that SIRT1 binds, deacetylates and inhibits p53 transactivation activity leading to its anti-senescent property. Subsequent studies identified that the anti-senescence effects of SIRT1 was a common feature of multiple cell types including human diploid fibroblasts (Huang et al. 2008), human umbilical vein endothelial cell line (Ota et al. 2007) and several cancer cell lines like breast cancer MCF-7, lung cancer H1299 and prostate cancer cells (Jung-Hynes et al. 2009; Ota et al. 2006). Importantly, SIRT1 is known to specifically repress genes involved in cell cycle arrest such as p16 (Huang et al. 2008, p. 21; Rathbone et al. 2008; Yuan et al. 2011, p. 27; Ota et al. 2006).

It is interesting to note that SIRT1 activity and/or levels have been proposed to decrease during aging in cells and mice (Yamakuchi et al. 2008). However, a clear picture that links the chromatin functions of SIRT1 and its role in cellular senescence is still not available. In support of such a role, reports that indicate chromatin relocalization of SIRT1 during aging imply a possible chromatin dependent effect of SIRT1 in aging/senescence (Oberdoerffer et al. 2008)**.** This finding is reminiscent of a similar phenomenon in yeast where the Sir2 redistribution on the genome has been observed in aging yeast cells (Gotta et al. 1997). It is clear that SIRT1 is important for the maintenance of telomeric chromatin in mammalian cell lines (Palacios et al. 2010). However, it is still not known if the functions of SIRT1 at the telomere are important for its role in cellular senescence. Further investigations are required to appreciate the link between Sir2/SIRT1 dependent global and/or locus specific chromatin changes and aging.

SIRT6 another important mammalian sirtuin has been clearly shown to play a major role in aging (Fig. 8.2). Mice deficient for SIRT6 exhibited progeroid symptoms (Kawahara et al. 2011) and results suggest that its ability to regulate DNA damage repair pathways were key to its role in aging (Mostoslavsky et al. 2006). Subsequently, SIRT6 was shown to deacetylate histone H3 at lysine 9 residue (Michishita et al. 2008), which incidentally is also targeted by SIRT1 (Vaquero et al. 2004). Reports that elucidated the ability of SIRT6 to regulate NF-kB dependent transcription showed that its role in aging is mostly determined by its ability to regulate inflammatory responses (Kawahara et al. 2011). It has been suggested that a dynamic relocalization of Sirt6 on chromatin is important for its ability to regulate organismal aging by controlling the expression of essential aging related genes, many of which are NF-kB targets (Kawahara et al. 2011). Further, SIRT6 has been shown to prevent telomere dysfunction in human cells by deacetylating H3K9 at telomeric loci, although, such an effect has not been observed in SIRT6 null mice (Michishita et al. 2008). Put together, it is evident that the functions of SIRT6 in mediating stress responses and at the telomere might have a bearing on aging and is probably dependent on its ability to deacetylate H3K9 residue. It is interesting to note that although both SIRT1 and SIRT6 have been implicated in similar pathways and at telomere functions, it is still unclear if they bring about a coordinated response to regulate aging.

8.3 Progeroid Syndromes and Chromatin

Progeroid syndromes are characterized by symptoms that mimic aging. Two of the most well studied clinical progeroid conditions are Werner syndrome and Hutchinson-Gilford progeria syndrome (HGPS). Werner's is a progeroid syndrome caused by mutations in the *WRN* gene, which encodes a member of the RecQ family of helicases. Intriguingly, some features of this disorder are also present in laminopathies caused by mutant *LMNA* encoding nuclear lamins A/C that causes HGPS. Recent studies suggest that epigenetic modifications in these progeroid genes lead to malignant transformation (Shumaker et al. 2006).

In HGPS, Lamin A gene is mutated resulting in a cryptic splice site in exon-11 causing 150 nucleotide deletion (LAΔ50). It was interesting to find that HGPS was associated with global changes in nuclear and chromatin architecture. Specifically, HGPS fibroblasts exhibit a loss of nuclear peripheral heterochromatin (Dechat et al. 2008), the severity of which depends on the accumulation of the abnormal LAΔ50 protein (Goldman et al. 2004). In addition, in cells derived from older HPGS patients, several heterochromatin marks, such as mono- and tri-methylated H3K9, show a dramatic decrease. A loss of the H3K27 tri-methyl mark was also observed in these cells and was correlated with a nine- to ten-fold decrease in the histone methyltransferase EZH2 expression. Another study, which looked at late-passage HGPS cells, observed an up-regulation of H4K20 tri-methylation (Shumaker et al. 2006). It is important to note that H4K20 tri-methylation has been shown to be

elevated in livers of older rats (Sarg et al. 2002) and in SAHFs in cultured cells (described above). The molecular mechanisms that link lamin A to heterochromatin formation are still not very clear. However, studies suggest that retinoblastoma protein (Rb) binds directly to type-A lamins (Ozaki et al. 1994; Johnson et al. 2004). Based on independent observations that Rb regulates histone methylation at H3K27, H3K9 and H4K20 residues, it has been speculated that Rb could be one of the factors that links aberrant histone methylation in HGPS (Blais et al. 2007).

Werner syndrome (WS) provides another example of a gene involved in aging and with tumor suppressor properties. As a result of the mutation in the *WRN* gene, cells from patients with WS show high genomic instability, especially at repetitive loci. But it is still not clear if WRN affects global chromatin that would eventually lead to aging. However, the role of chromatin in tipping the balance between senescence and cell proliferation becomes apparent from studies, which show that *WRN* is frequently repressed by CpG island hypermethylation in many human cancers (Agrelo et al. 2005).

8.4 Conclusion

Epigenetic marks, which are long lasting and inheritable, play a central role in mediating the outputs from the genome, in response to both extrinsic and intrinsic cues, and therefore, known to affect various biological processes. Hence, it is not surprising to find that chromatin is a major player in mediating cellular responses to aging. Although, all the critical chromatin components like DNA methylation, histone modifications and histone variants have been shown to be involved in this process, the mechanistic details that elicit these changes are less understood. Specifically, it will be interesting to address the cross-talk between classical aging pathways/mechanisms and chromatin signaling. It will be important to address if reversal of any of these chromatin changes would affect the aging process. In this regard, more work needs to be done on the role of chromatin modifiers, which mediate both global and locus specific effects on chromatin structure/function. Studies on proteins like HDACs and Sirtuins have indicated their involvement in the aging process. However, more insights into mechanistic details describing the chromatin effects are needed. Another important aspect that needs to be addressed is the apparent gaps in appreciating the roles of chromatin and chromatin modifiers in cellular and organismal aging.

Since aging is a complex biological process involving multiple factors, interventions aimed at one or more specific pathways/factors (to delay aging) are likely to give limited benefits. Targeting cellular components that would integrate the cues and the responses might turn out to be more beneficial. In this context, interfering with chromatin changes and/or chromatin modifiers that affect aging might become therapeutically relevant. This is crucial since small chemical modulators and/or dietary manipulations have shown promising results with regards to their ability to "delay the aging process" and are often mediated through some of these factors.

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Chapter 9
Homeotic Gene Regulation: A Paradigm for Epigenetic Mechanisms Underlying Organismal Development

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Abstract The organization of eukaryotic genome into chromatin within the nucleus eventually dictates the cell type specific expression pattern of genes. This higher order of chromatin organization is established during development and dynamically maintained throughout the life span. Developmental mechanisms are conserved in bilaterians and hence they have body plan in common, which is achieved by regulatory networks controlling cell type specific gene expression. Homeotic genes are conserved in metazoans and are crucial for animal development as they specify cell type identity along the anterior-posterior body axis. Hox genes are the best studied in the context of epigenetic regulation that has led to significant understanding of the organismal development. Epigenome specific regulation is brought about by conserved chromatin modulating factors like PcG/trxG proteins during development and differentiation. Here we discuss the conserved epigenetic mechanisms relevant to homeotic gene regulation in metazoans.

9.1 Animal Development

Embryonic development of diversified animals follows a common developmental theme. After fertilization, an embryo undergoes multiple rounds of cleavage to make embryonic cell mass. The ground plan for axis specification is laid down in an embryo early during embryogenesis. The molecular cues for dorso-ventral axis specification are found little later during cleavages. The most important event in embryogenesis in triploblastic organisms is gastrulation, when three germ layers, ectoderm, mesoderm and endoderm are formed. Each layer results in the formation of lineage specific organs and structures of the developing embryo. After crossing this crucial land-

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mark, an embryo starts transforming from the state of totipotency to specifying cell types, which is the concerted work of interconnected regulatory pathways.

Each organism has dorso-ventral, left-right and rostro-caudal axes. The marking of animal body plan onto early embryo is done with the help of few molecular cues known as morphogens, which form the gradient fields in the three-dimensional space of a developing embryo. These morphogens can be the class of transcription factors or the signaling molecules that ultimately set the hierarchy of developmental regulatory networks. In *Drosophila,* maternally deposited mRNA gradients of *bicoid* at the anterior end and *nanos* at the posterior end polarize the embryo and define A-P axis (Dahanukar and Wharton 1996; Driever and Nusslein-Volhard 1988a, b). Levels of these molecules across the A-P axis decide the further downstream activation of regulatory networks in a gradient specific manner. The specification of A-P body axis in vertebrates results from an interplay between the localization of Wnt, Fgf and retinoic acid, that are required for the specification of the posterior neural ectoderm (Iimura et al. 2009; Schier and Talbot 2005). Anterior axis is determined by the presence of an enzyme *cyp26* that degrades retinoic acid and hence specifies the expression of anterior genes forming anterior neural ectoderm (Kudoh et al. 2002). All vertebrate embryos have a convergent stage during embryogenesis wherein the rostral or head part becomes distinct and the neural tube extends towards the posterior end. Further along this axial structure, the segmentation of an embryo starts and the secondary limb bud field is specified from where appendages would arise subsequently, along the A-P axis. The part of the mesoderm, called paraxial mesoderm, extends posteriorly along with segmentation of an embryo into somites which gives rise to the vertebral column. The segmentation of an embryo simultaneously serves as the action ground for homeotic genes that will decide the identity of each segment. These genes are conserved across phyla and are instrumental for the diversification of morphological features along the A-P axis of bilaterians (Gaunt 1994; Holland et al. 1992; Krumlauf 1992).

9.2 Hox Genes

Hox gene products are transcription factors containing the conserved DNA binding homeo domain. This domain is 60 amino acids long and occurs in a conserved helix-turn-helix configuration for providing DNA target site binding specificity (Gehring and Hiromi 1986; Levine and Hoey 1988; Maconochie et al. 1996; Scott et al. 1989). Hox genes are organized in the form of clusters and this feature is conserved among all animals (Fig. 9.1). The Hox cluster of *Drosophila melanogaster* containing eight homeotic genes, is split into two complexes – the Antennapedia Complex (ANT-C) and the bithorax complex (BX-C) (Carroll 1995; Celniker et al. 1989; Maeda and Karch 2006). ANT-C consists of five homeotic genes, *labial (lab), proboscipedia (pb), Deformed (Dfd), Sex combs reduced (Scr)* and *Antennapedia (Antp).* The genes in this complex determine the head and first two thoracic segments (parasegment 1 to parasegment 5 in larval stages). The bithorax complex contains three homeotic

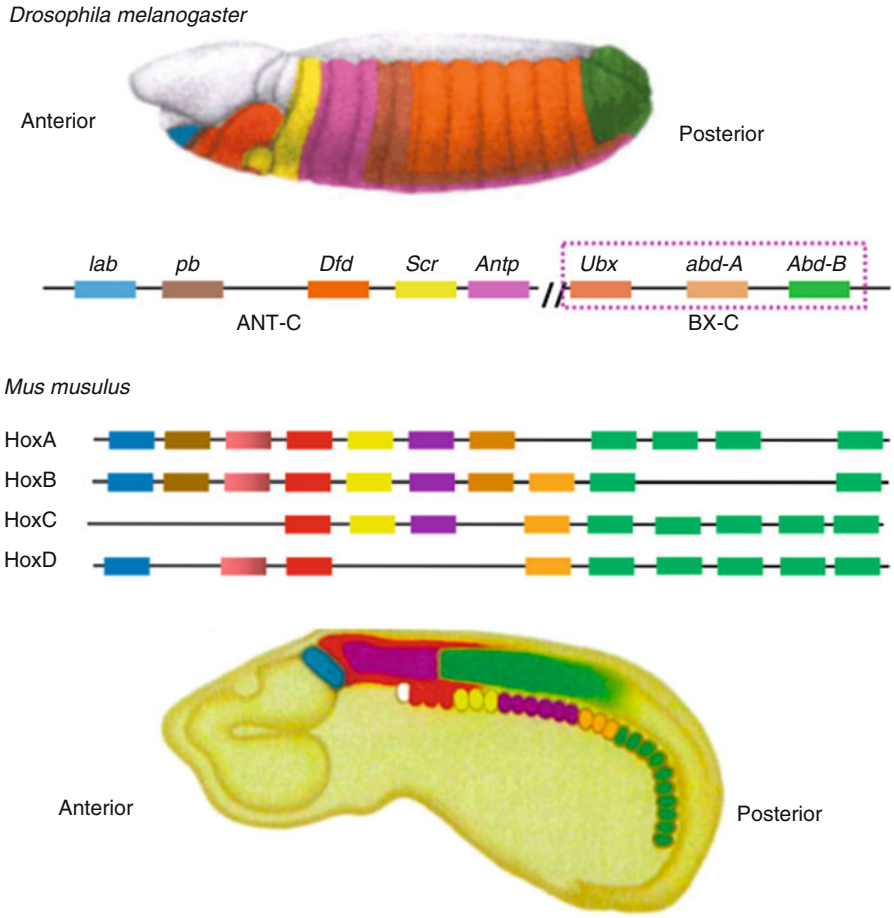

Fig. 9.1 Conservation of organization and expression of hox genes. Hox genes and their expression domains in *Drosophila* embryo (*top*) and mouse embryo (*bottom*) are shown. Flies have split hox cluster while mammals have four hox clusters. Each of the fly gene has one or more homologues in vertebrates. The posterior hox group (*Abd-B* group) in vertebrates has expanded, diversified and co-opted for limb formation. Hox genes and their expression domains are *color-coded* (Adapted from Vasanthi and Mishra 2008)

genes: *Ubx*, *abd-A* and *Abd-B*. These genes are responsible for the identity of parasegment 5 (PS5) to parasegment 14 (PS14) that form the third thoracic and eight abdominal segments in adults (Duncan 1987). There is a considerable level of structural homology between the various *Drosophila* hox genes and vertebrate homeodomain genes. In fact, a careful study showed that each fly hox gene has a corresponding homologue in vertebrates. The vertebrate homeotic complex has a total of 39 genes divided into four unlinked groups namely hox A, B, C and D complex. Each complex has few of the 13 paralogous genes missing in every organism arranged in spatially collinear manner (McGinnis and Krumlauf 1992). As depicted in the Fig. 9.1,

lab of fly corresponds to hox1, *pb* to hox2, *Dfd* to hox4, *Scr* to hox5, *Antp* to hox6, *Ubx* to hox7, *abd-A* to hox8 and *Abd-B* to hox9-13 paralogous groups in vertebrates (Maconochie et al. 1996). This classification is functionally supported by rescue of fly homeotic phenotypes by mouse hox genes (Lutz et al. 1996). The finding that hox genes are extremely conserved functionally and structurally was remarkable, and led to the theory of co-option of hox genes for conserved body plan.

9.3 Homeotic Transformations: Mutations and Phenotypes

William Bateson, in 1894, in his *'Materials for the Study of Variation, Treated with Especial Regard to Discontinuity in the Origin of Species',* first used the term 'homeotic' which is derived from the Greek word '*homeosis*', meaning 'becoming like' to explain the leg emerging in place of antenna of *Drosophila*. These kinds of dramatic changes, where one part of the body becomes like the other, were termed as 'homeotic transformations'. Since hox genes specify the identity of segments along the A-P axis, any mutation in them results in loss of segment identity of the corresponding gene expression. Moreover, any perturbation in the levels of hox gene expression results in the formation of confused segments, consequentially in anterior or posterior transformations. Experimental analysis of vertebrate hox code is done primarily in mice by gene targeting studies. It has been noticed that single hox gene mutant or knockout mice does not necessarily lead to homeotic transformations, suggesting that there is some level of redundancy in hox gene function. Triple homozygous knockout for hoxa10, c10 and d10 in mice show loss of lumbar vertebrae, instead, lumbar vertebrae take over the identity of thoracic segments with skeletal outgrowth as ribs. Similarly homozygous knockout mice for hoxa11, c11 and d11 have sacral vertebrae replaced by lumbar vertebrae. However, single paralogue knockout in mice for hox10 and 11 did not result in homeotic transformations (Wellik and Capecchi 2003). hoxd13 mutations in mice and in human are linked to problems in digit formation and urinogenital system (Fromental-Ramain et al. 1996; Goodman et al. 1998; Kondo et al. 1996; Muragaki et al. 1996). hoxb8 homozygous loss of function mutant mice do not show any transformations in skeletal elements, these mice have altered sensory neuron activity (Holstege et al. 2008). Behavioral analysis of these mice revealed that, they have obsessive-compulsive disorder of pathological grooming (Chen et al. 2010). These mice pull hair from their body and in the process have self-inflicted wounds. Mice transgenic for over expression of hoxb8 have mirror image duplication of posterior skeletal elements of limbs, like duplicated digits, carpals and ulna (van den Akker et al. 2001). Apart from assigning gross skeletal identity, maintenance of hox gene expression is necessary throughout the lifespan of an organism. Homeotic gene products target many downstream processes, which get abrogated upon loss of function or over expression. Upon RA exposure during early embryogenesis, anterior segments take the identity of posterior ones and hence there is loss of posterior segments resulting in shortening of the rostro-caudal skeletal axis. The effects of RA on murine vertebral transformations and hox gene expression have been extensively catalogued (Kessel and Gruss 1991).

9.4 Collinearity of Hox Genes and RA Induction

The remarkable feature of hox genes is that their expression pattern is dependent on the orientation they are arranged on the chromosome. The order of the arrangement of these hox genes on the chromosome exactly follows the order of expression pattern along AP axis of the embryo. Anterior hox genes (3′ of the cluster) are expressed in the rostral regions and posterior hox genes (5′ of the cluster) in caudal region of an organism. The correspondence of genomic arrangement with the domain specific expression in an embryonic space is called the 'spatial collinearity' (Krumlauf 1994). A distinguishing feature about vertebrate hox complexes is the correlation between the order of genes on the chromosome and time of expression of these genes, anterior genes being expressed early, and posterior genes being expressed later during axis formation and segment specification, this being termed temporal collinearity. The variations in spatio-temporal distribution of hox genes expression along the A-P axis can result in structural novelties in animal kingdom (Dekker et al. 1993; Duboule 1994; Gaunt and Strachan 1996; Kondo et al. 1998). In essence each segment in the body is dependent on 'hox code' for its identity and segment specific variations can occur as a result of subtle variations in this 'hox code'.

During somitogenesis the oscillating gradient of retinoic acid (RA) along with Wnt/Fgf signaling is crucial for segmentation of an embryo. In limb bud formation, RA polarity is necessary for maintaining the zone of polarizing activity (ZPA) and thus activation of region specific induction of separate developmental pathways (Buxton et al. 1997; Stratford et al. 1996). RA has morphogenetic properties; the dose of RA along the A-P axis is crucial in maintaining the hox gene expression in correct order. Retinoic acid induces dose dependent hox gene expression in a collinear fashion. High doses of RA lead to induction of posterior hox genes earlier than specified and thus results in axial malformations. This results in loss of posterior structures of the embryo (Kessel and Gruss 1991). Nevertheless RA acts as morphogen but in higher dosages can lead to severe developmental problems. Within the developing embryo peri-nodal tissue cells surrounding the hensen node, mesodermal floor plate and later on limb buds are endogenous source of retinoids (Horton and Maden 1995; Maden et al. 1988). As the embryo axis elongates, endogenous retinoid production also increases in the embryo (Hernandez et al. 2007).

It is known that anterior hox genes need less RA than posterior hox gene for activation and thus setting up progressively, an increasing front of RA in the elongating embryo (Conlon 1995). Levels and gradients of retinoic acid in an embryo are maintained by its *Raldh2* dependent biosynthesis, and *cyp26* dependent degradation. Embryos deficient in both the pathways result in disturbed axial patterning. Mouse embryos developed in vitamin A deficient mothers show pleiotropic developmental defects, which are also observed upon vitamin A deficiency in vertebrate model systems and are classified as VAD syndrome (Mark et al. 2004, 2006). RA is instructive during hox gene specification of the metameric somites and induces limb bud formation and organogenesis.

9.5 Regulation of Master Regulators

Homeotic genes expression follows a spatio-temporal manner but the understanding of how these genes are regulated to execute the 'hox plan' is still developing. The levels and the kinds of *cis* and *trans* regulatory inputs required by hox genes to bring about the read out in the form of expression pattern in a developing embryo are (may be) enormous and complex. By employing novel strategies of regulating genes such as promoter sharing, promoter competition, enhancer sharing, enhancer selectivity, insulator elements, silencers, repressors, maintenance elements, Retinoic acid response elements (RAREs), long range interactions, higher order chromatin re-organization and non-coding RNAs, homeotic clusters have become the paradigm to understand different ways and complexity of gene regulation in the past three decades.

9.5.1 Features for Tight Regulation of Hox Clusters

The tight association of clustered hox genes is conserved during evolution and necessary to establish hox expression domains in proper space and time during embryo development. It has been shown that a gene from one paralogous hox group can overtake the function of the other, like hoxa1 insertion in place of hoxb1 deletion rescues the hoxb1 phenotype. But when the hox gene position is changed along the cluster such that it resides in non-native regulatory context, its expression is governed by neighbouring regulatory elements. For example, hoxd9-LacZ transgene when placed posterior of the hox cluster, starts expressing in spatio-temporal domain of hoxd13, similar is the case when hoxb1-LacZ transgene is inserted in the posterior locations (Kmita et al. 2000). These experiments clearly illustrate that hox clusters retain the spatio-temporal information for tight regulation in their non-genic region. Any perturbation, for example inversions or duplications or deletions in the organization of the cluster lead to disturbed homeotic gene expression. Many of the homeotic mutations in *Drosophila* mapped to regulatory sequences rather than the coding region of the hox genes (Bender et al. 1983). Homeotic transformations may also result by mutating certain factors, which are *trans*-regulators of homeotic complexes. Expression and regulation of the hox cluster requires both *cis* and *trans* inputs for its initiation, modulation and maintenance in correct space and time.

9.5.2 Cis-Regulatory DNA Elements in Hox Clusters

Eukaryotic genome is dominated by non-coding sequences in its content, while coding (genic) sequence is a small minority, only 2% of the genome. For example in human, to control and modulate this 2% of the genome, sequences of non-coding potential are employed and incorporated in the genome, keeping the coding process

itself conserved (Flam 1994). Homeotic genes exist in clusters along with their *cis*-regulatory DNA elements present within or in the flanking regions of the cluster making up the 'homeotic-landscape' (Duboule 2007). Different kinds of *cis*-regulatory modules are juxtaposed in the hox complex to switch on and off, or even to command the level of gene expression in spatially and temporally distinct domains. Most of these *cis* elements are indentified by deletions and are modular in nature as assayed in transgenic context.

9.5.2.1 Enhancers

Several *cis*-elements having enhancer function have been found in vertebrate hox clusters. Apart from enhancing the level of expression of the gene in a tissue specific manner, enhancers in hox clusters have evolved with the need to follow the 'hox clock'. Early expression of hoxc8 is associated with a conserved upstream element having enhancer function. Deletion of the hoxc8 enhancer results in anterior transformations in axial skeleton associated with the delayed expression of hoxc8 (Juan and Ruddle 2003). Deletion of hoxd11 enhancer is responsible for the delayed hoxd11 expression resulting in homeotic transformations in the sacral region of the axial skeleton (Gerard et al. 1997; Herault et al. 1999; Zakany et al. 1997).

hoxb3 and hoxb4 have subset of expression domain, which is overlapping in rhombomere6/7 of hindbrain. This sharing of domain in space is a result of sharing of common enhancer CR3, present in the intergene of hoxb3 and hoxb4. hoxb4 and hoxb5 gene products can auto-regulate and cross-regulate respectively the enhancer by direct binding, unlike hoxb3 protein. This CR3 region in transgenic *Drosophila* can also be activated by *Dfd,* which belongs to hox4 group of genes (Gould et al. 1997; Sharpe et al. 1998). This cross-species conservation in *enhancer sharing* and *auto-regulative* mechanisms highlight the evolutionary constraints imposed upon regulation of hox clusters. hoxb4 and hoxb5 intergene has an atypical shared enhancer element, having neural and limb enhancer modules. In transgenic assay, limb enhancer region can drive only hoxb4 promoter but not hoxb5 promoter, suggesting the *enhancer selectivity*. Another module of the same enhancer has a neural enhancer region, which can drive reporter expression equally from hoxb4 and hoxb5 promoters in transgenic assay. However, when placed in dual reporter construct, the neural enhancer has preference towards hoxb4 promoter. hoxb4 promoter competes for the enhancer, but this *promoter competition* is abolished when distance between hoxb4 promoter and neural enhancer is increased, and neural enhancer starts driving the hoxb5 promoter instead, due to close proximity with the latter (Sharpe et al. 1998). The tight placement of enhancers within the hox cluster is highly context dependent making them more robust and innovative in performance.

Regulation of posterior hoxd genes depends on the large global control region (GCR) identified 240 kb upstream of the HoxD complex having enhancer like function. This GCR is described as a 40 kb region with several regulatory domains. Locus specific control of posterior hoxd genes is governed by GCR, which when deleted, abolishes the expression of posterior hoxd genes in the limb region. Serial deletion

and duplication studies on posterior hoxd genes implicate the mechanism of GCR sharing among posterior hoxd genes for 'quantitative collinearity' in limb bud. For example deletion of hoxd13 and hoxd12 genes results in hoxd13 like expression of next posterior gene i.e., hoxd10. This analysis conclusively suggests that global enhancer discriminates its sharing depending upon the location in the posterior HoxD complex and thus forms the quantitative collinear gradient of posterior hoxd genes in the limb region (Kmita et al. 2002a). The long range interaction of enhancer like locus control region is a vertebrate innovation typically to regulate clustered genes.

9.5.2.2 Boundary Elements

On *prima facie,* enhancers in hox clusters are responsible for distinct domain specific expression of hox genes along the body axis, but essentially, enhancers are capable of promiscuity in driving the expression of nearby genes too. Nevertheless, enhancer selectivity and preference has been observed in transgenic assays, activity of enhancers alone cannot justify the tight regulation operating in hox clusters. Certain *cis*-regulatory elements restrict the illegitimate enhancer-promoter crosstalk exist in the genome and thus demarcate functional chromatin domains. Moreover, apposing functional domains within hox clusters demand existence of higher order insulation mechanisms to prevent spread-over of adjacent enhancer action. Boundary elements are modular and can be assayed in transgenic context as blockers of enhancer-promoter interaction. Essentially, boundary or insulators protect genes from flanking chromatin environment but do not by themselves instruct the transcriptional state of a gene.

Hox complex has closely spaced differentially expressed hox genes and boundary elements are required to maintain separate expression units as functional chromatin domains. Dissection of the regulatory landscape of *Drosophila* BX-C complex has provided ample evidence to prove that insulators are critical in defining domain specific hox expression. Segment specific expression pattern of *Ubx*, *abd-A*, *Abd-B* is controlled by a large *cis*-regulatory region, ~300 kb (Karch et al. 1985), that is subdivided into nine functionally autonomous *cis*-regulatory domains *(abx/bx, bxd/pbx*, *iab-2* to *iab-8*). Each *cis*-regulatory domain directs expression of homeotic genes, *Ubx*, *abd-A*, *Abd-B* in a specific parasegment. For example, *iab-5 cis*-regulatory domain controls *Abd-B* expression pattern in parasegment10 (PS10)/A5. Similarly *iab-6*, *iab-7* and *iab-8* control *Abd-B* expression in PS11/A6, PS12/A7 and PS13/A8 identity respectively (Boulet et al. 1991; Celniker et al. 1989, 1990). Genetic and molecular studies have identified chromatin domain boundaries that demarcate the *cis*-regulatory domains that ensure the functional autonomy of each regulatory domain (Mihaly et al. 1998). For example in PS11, *Fab-7* boundary protects active *iab-6 cis*-regulatory domain from inactive *iab-7* domain restricting inappropriate regulatory interactions between the two domains. Deletion of *Fab-7* boundary allows the next *cis*-regulatory domain, *iab-7* to drive *Abd-B* in PS11 resulting in A6-A7 transformation in adult fly.

In vertebrate hox clusters, the existence of insulator elements is expected, but so far only one such element has been identified. The boundary element of hox clusters that is genetically well dissected is between Evx2-hoxd13. Both Evx2-hoxd13 have

a common regulatory network and are under the influence of GCR. Neural expression of posterior hoxd genes is supposedly prevented by Evx2-d13 boundary element (Kmita et al. 2002b). In order to test boundary function effectively, several regions of evx2-d13 intergene were tested by placing it towards the 3′ of Evx2 gene tagged with lacZ gene as reporter. A region close to Evx2 promoter harboring boundary activity, when placed downstream to the Evx2 could block neural enhancer from driving Evx2 expression in CNS (Yamagishi et al. 2007). When tested in *Drosophila* and mammalian K562 cells, Evx2-d13 core boundary function could be narrowed down to a 3 kb region harboring binding sites for *Trl*/GAF. The Evx2-d13 boundary needs *Trl*/GAF for its insulation activity (Vasanthi et al. 2010). The *Drosophila* homologue of Evx2 is *even-skipped* that also has promoter associated *Trl* dependent boundary function (Ohtsuki and Levine 1998). The *Trl* dependent insulation mechanism of Evx2-d13 boundary is reminiscent of *Drosophila* hox regulation although there is no sequence similarity whatsoever. Smaller fragments of 1 kb derived from bigger 3 kb Evx2-d13 boundary are capable of enhancer blocking in *Drosophila*, however they are weak blockers. Evx2-d13 boundary model illustrates that boundary function in vertebrate hox cluster is spread-over a few kilobases unlike much smaller boundaries of *Drosophila* BX-C. This may indicate recruitment of additional regulatory motifs in vertebrate boundaries.

The sequence comparison of various boundary elements has failed to identify any significant homology except for small conserved sequence motifs of unknown significance or stretches of AT rich regions (Karch et al. 1994; Vazquez et al. 1993). Chromatin domain boundaries that subdivide the genome into functional units have been isolated using different criteria (West et al. 2002). Interestingly, it has been observed that there is a great degree of conservation of the boundary activity across the species (Chung et al. 1993). This could be because of the conservation of the factors associated with the boundary elements. For example, 5′ HS4 boundary element from mouse *β globin* locus acts as strong boundary in the enhancer blocking assay system in *Drosophila.* On the other hand, *Fab-8* boundary element from the *Drosophila* bithorax complex functions as an enhancer blocker in vertebrate cells (Moon et al. 2005).

The mechanism of boundary elements function is largely unknown, though there are several largely speculative models to explain it. (Capelson and Corces 2004; Kuhn and Geyer 2003; Raab and Kamakaka 2010; Valenzuela and Kamakaka 2006). Boundary elements may function in combination with nuclear matrix through SAR/MAR (Scaffold Associated Region/Matrix Associated Region) like elements where the insulating effects are a consequence of this association (Gerasimova and Corces 2001; Pathak et al. 2007). Another way boundary elements might function is by inhibiting the interaction between the promoter and the signal from enhancers (Gaszner and Felsenfeld 2006). One more way in which boundaries might act is by competing with promoter to capture enhancer, namely the promoter decoy model (Dorsett 1999; Gause et al. 2001). Recently, it has been shown in *Drosophila* that the most plausible action of boundary elements is by looping DNA (Gohl et al. 2011). The boundary action of an element is dependent on the kind of other boundary elements flanking the transcriptional unit. Almost all reports of boundary elements discuss possible mechanisms of action but no single model explains fully all the

aspects of their action, possibly because more than one mechanism are involved (Iqbal and Mishra 2007; Mishra and Karch 1999).

9.5.2.3 Retinoic Acid Response Elements

Hox gene activation is critically responsive to retinoic acid (RA) within the developing embryo. RA is present in a concentration gradient and thus acts as a morphogen for pattern formation and cell differentiation. Retinoic acid works as a ligand for retinoic acid receptors (RARs) which upon binding to cis-elements called Retinoic acid response elements (RAREs) present in gene promoters brings about changes in transcriptional state of the gene. Exposure of a developing embryo to RA results in pleiotropic effects like perturbations in axial patterning, limb bud formation, segmental identity and numbers of metameric structures along the A-P axis.

In the absence of RA ligand (or in the presence of antagonist ligand) binding domain of RAR/RXR heterodimer recruits co-repressor complex NCoR/SMRT and maintains repression (Leid et al. 1992). Upon RA (agonist ligand) binding, there is a conformational change in the ligand-binding domain at the surface of heterodimer RAR/RXR allowing the binding of coactivators (p300/pCAF complex) with subsequent release of corepressor complexes (Glass and Rosenfeld 2000). Null mutants of these receptors show equally pleiotropic effects like VAD deficiency including disturbed hox gene expression and axial skeletal defects (Kastner et al. 1995; Mark et al. 2009). The release of NCoR/SMRT complex from RARE triggers JMJD3, a H3K27 demethylase, to remove the H3K27me3 repressive mark (Jepsen et al. 2007). RA induced de-repression is accompanied by concomitant release of SUZ12 and erasure of the H3K27me3 mark which essentially puts RA in a more instructive role to remove inactive marks (Gillespie and Gudas 2007). Upon removal of RA treatment SUZ12 re-associates with RARE. RA mediated chromatin restructuring to lift PcG repression from hox genes is shown via another H3K27me3 demethtylase UTX, which even dislodges EZH2 from repressive domain (Agger et al. 2007; Lee et al. 2007). UTX mediated demethylation is thought to ensure quick removal of repressive mark and hence activation in a precise spatio-temporal window. Removal of the H3K27me3 mark from hoxa1 RARE that is 4.6 kb away from hoxa1 promoter, results in increased occupancy of RNAPolII at promoter region. Concomitant increase in H3K4me3 mark in hoxa1 and hoxb1 RARE upon RA exposure does not increase the MLL complex occupancy at these loci pointing to an MLL independent mechanism operating for assignment of activated state (Kashyap et al. 2011).

9.6 PREs – Polycomb Response Elements

With an identical genome, metazoans know the art of making different cell types. Hox genes define and specify the transit of plain embryo to a working ensemble of specified cells and structures. These gene products are quite authoritative in specifying

cells to differentiate and follow a particular lineage and this specification of cellular identity has to be memorized by the cell type for the remaining lifetime of an animal. Maintenance of the expression states of crucial cell-identity specifying genes is controlled by robust cellular system comprising conserved *Polycomb/trithorax* group (PcG/trxG) of proteins. These proteins are in-fact so important throughout the lifespan of an animal that loss of PcG/trxG system any time creates total chaos in otherwise well disciplined cell type specific gene expression. Expression of hox genes is maintained by two antagonistic sets of these genes: *Polycomb* group (PcG) and *trithorax* group (trxG) of genes (Kennison 1995). The PcG and trxG of proteins were first identified as *trans*-acting regulators of homeotic genes. The phenotypes observed in case of these genes are similar to the homeotic phenotypes. Suppressors of the PcG phenotype have been identified that fall into *trxG* genes. Several *PcG* and *trxG* genes have been identified in *Drosophila.* Molecular analyses of these proteins showed that they might act in large complexes and modify the local properties of chromatin to maintain transcriptionally repressed or active state of the target genes. PcG proteins target several genes involved in signaling pathways that are conserved, suggesting that these proteins might act as regulators to coordinate a variety of developmental and differentiation processes; consistent with recent evidences suggesting that the function of the conserved PcG/trxG complexes is dynamically modulated in time and space (Klebes et al. 2005; Lee et al. 2005; Maurange et al. 2006). PcG/trxG proteins interact with specific *cis* regulatory elements called *Polycomb* Response Elements (PREs) (Chan et al. 1994; Christen and Bienz 1994; Simon et al. 1993) and are conserved across the species (Ringrose and Paro 2004).

Three crucial steps required to confer Polycomb dependent cellular memory module are PcG targeting to specific DNA, chromatin re-structuring corresponding to the expression state of loci and memorizing it through cell divisions. These steps are the hallmarks of an element responsive to PcG/trxG regulation and thus several assays to identify PRE (Polycomb response element) are designed accordingly.

9.6.1 Targeting of PcG/trxG

Cis-regulatory elements responsive to PcG/trxG systems are called Polycomb Response Element (PRE)/Trithorax Response Element (TRE), having the repressor and activator function respectively. However due to the overlapping nature of existence of these two elements it is sometimes tedious to functionally separate them out.

Polycomb mediated silencing is the action of three repressive complexes, 3MDa Polycomb Repressive Complex 1, PRC1 and 600 kDa, Polycomb Repressive Complex 2, PRC2, which has H3K27 tri-methyltransferase activity and PhoRC (PHO dependent repressive complex) (Table 9.1). Targeting of these complexes to PRE is known by PHO, PHO-like, Trl-GAGA, Pipsqueak, DSP1 (Protein dorsal switch 1), SP1 (specificity protein 1), Grainy head and ZESTE in *Drosophila*. In vertebrates, YY1 (Yin-Yang 1, homologue of PHO) and ThPOK (homologue of Trl-GAGA) (Matharu et al. 2010), are involved in PcG targeting, while other

Table 9.1 PcG complexes: fly genes and vertebrate homologues

Complex	Fly	Vertebrate homologues
PRC1	PC	CBX2, CBX4, CBX6, CBX7, CBX8
	PSC	PGF6 (MBLR), PGF (BMI1), PGF5, PGF3, PGF2 (MEL18), PGF1 (NSPc1)
	PH	PHC1, PHC2, PHC3
	SCE	RING1, RING2
	E(Z)	EZH1,EZH2
	ESC/ESCL	EED1, EED2, EED3, EED4
	SU(Z)12	Suz12
	NURF55	RBAP48, RBAP46
	PCL	PCL1 (PHF1), PCL2 (MTF1), PCL3 (PHF19)

homologues are not yet identified (Beisel and Paro 2011). Mutations in these proteins result in loss of PcG silencing, which suggests that sequence specific targeting of PcG is crucial for further PcG repression. YY1 knockdown in mouse myoblasts prevents binding of PRC2 member EZH2 (enhancer of zeste homologue 2) and thus loss of H3K27 tri-methylation (Caretti et al. 2004). It is difficult to predict PRE due to lack of sequence homology, therefore many bioinformatics tools have been designed to predict the binding motifs for these DNA targeting proteins (Ringrose and Paro 2004; Ringrose et al. 2003). Several ChIP based studies for PcG targets in mammals revealed close association to the target promoters (Boyer et al. 2006; Bracken et al. 2006; Endoh et al. 2008), while in ES cells this targeting is restricted to promoters with high CpG content (Ku et al. 2008). There are only three PREs so far identified in higher vertebrates. In mouse PRE-kr is identified for regulating *MafB* gene, which has YY1 and GAGA binding sites (Sing et al. 2009). Another mammalian PRE identified so far is HoxD11.12 PRE, which again has YY1 binding motifs (Woo et al. 2010). Up to 25 kb upstream to mouse HoxD cluster a fragment initially identified as a repressor element (Kondo and Duboule 1999) by deletion analysis was later shown to recruit EED (PRC2 member) in a temporal fashion for silencing mechanisms (Mishra et al. 2007). This fragment is shown to have repressor function in transgenic *Drosophila* along with eye color variegation and PC recruitment. Targeting of PcG/trxG to PRE thus satisfies one of the criteria for a functional PRE.

Recruitment of trxG is somewhat more complex and less understood than PcG complexes. At least four different complexes containing trxG proteins have been identified from *Drosophila* embryos: SWI/SNF complex (has Brm, Osa, Moira, Snr1 that catalyses ATP dependent chromatin remodeling), NURF complex (has Iswi, N38, N301, N55 that catalyses ATP dependent chromatin remodeling), TAC1 complex (has Trx, dCBP, Sbf1 has SET domain proteins involved in methylation of histones) and Ash1 complex (Ash1, dCBP has SET domain proteins involved in methylation of histones). Many *trithorax* homologues have been discovered in vertebrates, having conserved function as in *Drosophila*. Binding of such complexes has been shown at hox genes but the mechanism of activation is not fully understood (Hsieh et al. 2003; Hughes et al. 2004).

9.6.2 PcG/trxG Dependent Chromatin Modulation

Chromatin regulators such as PcG/trxG directly command the gross restructuring of the chromatin and have an effect on accessibility of the transcriptional machinery. Histone methyl-transferase (HMT) activity of the PRC2 complex resides with E(Z) of flies and EZH2 of mammals. It needs the assembly of core PRC2 complex members, Suz12, and ESC (in flies)/EED (mammals) for its catalytic activity. Tri-methylation of histone 3 at lysine27 strictly tracks with the occupancy of PcG complex while H2K27 mono and di-methyl marks are widespread in the genome but only the former being involved in the PcG dependent silencing of chromatin. The complexity of the PRC2 complex is not yet properly understood. Some studies suggest that certain isoforms of EED (homologue of fly ESC) can methylate H1 linker also but whether this methylation is necessary for PcG silencing is not known (Martin et al. 2006). Another homologue of E(Z) in mammals, EZH1, is expressed in adult non-dividing tissues having less HMTase activity than EZH2 which is crucial for embryogenesis and cell differentiation (Martin et al. 2006). PRC1 complex recognizes the H3K27 modification for PRE targeting, loss of PRC2 leads to loss of PRC1 targeting also. Another complex PC-like (PCL) in flies and PHF1 in mammals is required to enhance the HMTase activity of PRC2 complex. In flies PCL recruits PRC2 complex (Savla et al. 2008) and thus loss of PCL leads to de-repression of some of the target loci but not all. However, this sequence of PRC2 and PRC1 targeting is still debatable because of a line of evidence cited which show PRC1 occupancy and H3K27 tri-methylation to be exclusive at certain loci in *Drosophila* cells (Schwartz et al. 2006). Studies like PHO mediated targeting of the PRC1 complex (Mohd-Sarip et al. 2006) and retention of PRC1 upon reduction of H3K27 tri-methylation at few of the target loci support the targeting of PRC1 independent of H3K27 tri-methylation (Schoeftner et al. 2006). But at many developmentally important loci studied so far PRC1 occupancy coincides with the H3K27 mark and further leads to chromatin modulation such as to impede RNA PolII machinery to establish silencing (Chopra et al. 2009). However recent studies are pointing towards PC mediated long-range interaction of distant Pc sites to assemble in one compartment and thus facilitate the spreading of silencing machinery over large loci (Bantignies et al. 2011; Lanzuolo et al. 2007; Tiwari et al. 2008). This recent school of thought is developing to address the recruitment of PRC1 complex independent of PRC2 for silencing during X-inactivation also (Casanova et al. 2011; Tian et al. 2010; Zhao et al. 2010). The mechanisms of PcG dependent silencing looks simple in *Drosophila* which has limited complexity in PRC1 and PRC2 members, unlike in the case of mammals where the repertoire of Pc family itself is expanded (Table 9.1).

9.6.3 Bivalent Chromatin Domains: PcG/trxG

Since PRE/TRE exist in union many a time, the chromatin domains flanking this common fragment have differential transcriptional information (Fig. 9.2). The mechanisms of PcG silencing and trxG activation are interdependent and their

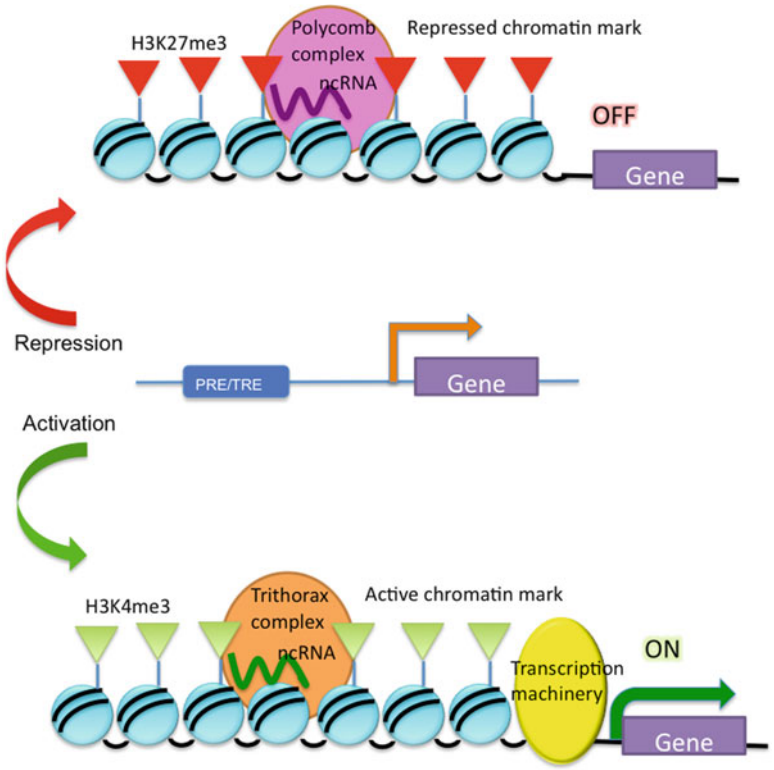

Fig. 9.2 PcG/trxG complexes and chromatin modification. Schematic representation of PcG and trxG complexes associated on PRE/TRE. PcG and trxG complexes deposit histone marks that play complementary roles in silencing and activation of their target chromatin. A developmental signal determines whether the PRE/TRE mediates gene activation or gene repression, which is accompanied by histone H3K27me3 and H3K4 demethylation or H3K4me3 and H3K27 demethylation, respectively. PcG/trxG complex can form 'bivalent chromatin domains' by having adjacent active and silenced chromatin mark. PcG/trxG complexes interact with non-coding RNAs, which helps in recruitment. PRC complex leads to histone modification H3K27me3 causing local silenced chromatin to be silenced. On the contrary for active chromatin, trxG complexes cause H3K4me3 modification and thus decides recruitment of transcription machinery for expression of genes

assembly on chromatin is also concerted. *Drosophila* PREs are known to have distinct TRE fragment, for example *bxd*-PRE regulates *Ubx* and has dissectable TRE (Tillib et al. 1999). This makes sense because the Ubx gene has to be switched on and off in a spatial manner during development. PcG takes care in the domain where the *Ubx* expression has to be silenced and trxG where it has to switch on. This PRE/TRE switch is common feature in *Drosophila* hox complex while in mammalian development this possibility has not yet been explored. However, a study in mice showed antagonist roles of Bmi1 (PcG member) and Mll (trxG member) during regulation of hox genes. *Bmi1* mutant showed posterior transformations of cervical vertebrae, which got corrected to certain extent in the background of *Mll*

heterozygous mutation. HoxC in the same study was analyzed as target locus for Bmi1 and Mll. Loss of Bmi1 results in up-regulation and loss of Mll results in down-regulation of hoxc8 in mice (Hanson et al. 1999).

Histone modifications studied across developmentally important loci in ES cells revealed 'bivalent domains' having H3K27me3 and H3K4me3 at promoters (Bernstein et al. 2006) (Fig. 9.2). Genome-wide co-occupancy of H3K27 tri-methylation and H3K4 tri-methylation in ES cells found at many promoters is either bound by both PRC1 and PRC2 or only PRC2 (Ku et al. 2008). However PRC1 occupied bivalent domains are more stable and retain silenced information after differentiation. 'Bivalent chromatin domains' occupied by PRC1 and PRC2 have transcriptionally poised Ser-5 phosphorylated RNA PolII. Loss of any PRC1 or PRC2 member results in firing of transcription by RNA PolII at these promoters. H3K4 demethylase, Jarid2, associates with PRC2 components and necessary for poising of RNA PolII. (Landeira et al. 2010; Li et al. 2010; Pasini et al. 2010). Intriguingly, Jarid2 depletion results in loss of poised RNA polII with lower transcript levels as compared to even wild type ES cells and hence paused differentiation (Landeira et al. 2010). Another demethylase, RBP2 (retinoblastoma binding protein 2-H3K4 demethylase) has been shown to localize with PRC2 complex suggesting robust silencing by PRC2 targets. Although the interaction is transient, it is functionally relevant. During hox gene activation RBP2 dislodges from transcriptionally poised promoters, and again is required to silence it after differentiation (Pasini et al. 2008).

Another class of demethylases for H3K27 is also implicated in ES cells differentiation and embryonic development. UTX and Jmjd3 associate with Mll2/3 trithorax complex and removes H3K27me3 repressive mark. UTX acts on many hox promoters to remove the repressive mark, which are subsequently marked with H3K4me3 active mark upon RA signaling (Lee et al. 2007). Knock down of UTX in zebrafish embryos shows anteriorization phenotype with posterior shift of hoxc8 anterior expression boundary and reduced transcription of posterior hox genes (Lan et al. 2007).

Zygotic genome remains inactive till maternal-zygotic transition. Out of histone3 lysine modifications, H3K27me3 and H3K4me3 marks are detected only after maternal-zygotic transition in zebrafish embryos (Vastenhouw et al. 2010). The embryonic pluripotency coincides with the arrival of these two marks in the zygotic epigenome. The existence of bivalent domains corroborating the ES cells studies is suggested during zebrafish embryogenesis.

9.6.4 H2A Ubiquitination and PcG Silencing

Another covalent histone modification found to be involved in PcG mediated silencing is H2AK119 ubiquitination (H2Aub) (Shilatifard 2006). Loss of H2A ubiquitination from PcG target promoters dislodges PRC1 complex but H3K27me3 mark remains. RING1 and BMI1 (PSC in flies) members of PRC1 are part of this H2Aub complex named PRC1-L (PRC1-like)/dRAF (dRing associated factors) in flies/BCOR (BCL6 CoRepressor) complex in mammals (Table 9.1). PRC1-L complex can ubiquitylate

H2A at lysine 119. Loss of dRING in *Drosophila* leads to PcG mediated derepression of *Ubx* concomitant with the loss of H2Aub (Wang et al. 2004). Interestingly PRC1-L complexes contain KDM2 (a histone lysine demethylase) as one of the component, which has been shown to have high ubiquitinating activity at PcG targets. KDM2 demethylates H3K36me2, an active mark, and facilitates H2AK119 ubiquitination. *Drosophila* KDM2 is found to be the enhancer of PcG phenotype and suppressor of trxG phenotype (Lagarou et al. 2008). Loss of H3K27me3 demethyalse, UTX, results in increased ubiquitination of H2AK119 at several hox promoters, which reinstates the role of H2Aub in PcG silencing. H2A de-ubiquitinating complex (PR-DUB-Polycomb repressive deubiquitinase) contains *Calypso* and ASX, mutants of which show de-repression of PcG targets (Scheuermann et al. 2010). Interestingly these mutations also show slight increase in H2B ubiquitination along with H3K4 methylation, both being associated with active mark. H2Bub is shown to be important for sub-telomeric anti-silencing (Wan et al. 2010), however its role in PcG silencing or trxG activation is yet to be deciphered. ZRF1 (zuotin-related factor 1) in mammals is recruited to H2Aub and displaces PRC1 complex during differentiation (Richly et al. 2010). ZRF1 is required for retinoic acid dependent transcriptional activation and H2A de-ubiquitination of hoxA promoters. Ubp-M (Usp12) has the activity of H2A specific de-ubiquitination, abrogation in Ubp-M function results in repressed hoxd10 and abnormal posterior development of *Xenopus* (Joo et al. 2007). H3K27me3 is required to target PRC1 but considering another layer of histone modifications operating to stabilize PRC1 suggest that H3K27me3 mark is surely not enough to hold PRC1.

9.6.5 Elongation Block and PcG Silencing

PcG/trxG co-existence assures the cell to execute developmental processes in time and in an energy efficient manner. At the core of gene regulation the transcriptional machinery has to switch ON/OFF. PcG mediated chromatin marks were thought to be permanent previously but in the light of new studies it seems to be more dynamic. PcG silencing correlated for 'transcriptionally inactive chromatin' is now taken as 'transcriptionally poised chromatin'. It has been shown that *Ubx* and *AbdB* contain paused RNA polymerase when silenced. Co-occupancy of stalled RNA PolII along with PcG members at hox promoters (Chopra et al. 2009) is explained as the cellular mechanism to tilt the balance to ON/OFF expression state whenever required. Many 'bivalent' domains in gene promoters show the presence of stalled RNA PolII (has phosphorylated at Ser5) and these genes also contain H2Aub marks. Depletion of RING1 releases this elongation block by having RNA PolII phosphorylated at Ser2 (Stock et al. 2007). Another line of study about H2A ubiquitination suggests that NcoR (nucleosomal co repressor factor) dependent H2Aub activity is necessary for stalled RNA PolII, which prevents the recruitment of FACT complex and hence blocks elongation (Zhou et al. 2008). Conversely H2B ubiquitination associates with FACT complex on actively transcribing promoters (Minsky et al. 2008).

Studies independent of PcG mechanisms also point towards elongation switch for regulation of gene expression. Promoters of developmentally important genes in ES cells having actively marked nucleosomes, H3K4me3/H3K9Ac along with RNA PolII undergo transcriptional initiation, but only subset of genes are fired for elongation (Guenther et al. 2007). Trithorax members are known facilitators for this elongation to overcome PcG repression.

9.6.6 Non-Coding RNA in PcG/trxG Regulation

Nearly 98% of the eukaryotic genome is non-coding, and new techniques for genome level sequencing have shown that most of the non-coding genome is being transcribed. Emerging roles of non-coding RNAs in the genome suggest that they constitute another layer of regulatory networks operating in the genome. Targeting of PcG/trxG has been shown to be dependent on either *cis* or *trans* non-coding RNA transcription. Both in flies as well as in mammals non-coding RNAs, sometimes in both the orientations are detected through PRE/TRE, which can also directly bind to PcG/trxG complex (Fig. 9.2). These can be few nucleotides (100–200 bp) to few kilobases in length. A non-coding RNA of 26 kb length has been detected through fly *bxd*-PRE, which is further processed (Lipshitz et al. 1987). There is no general rule as of expression domain of these non-coding RNA with that of its cognate hox gene expression state. However these ncRNAs are expressed before the appearance of hox gene mRNA, setting up the stage for 'PcG or trxG recruitment' depending upon the regulation required in spatio-temporal context. This kind of PcG/trxG recruitment to PRE/TRE by ncRNAs is thought to be sequence dependent for exact targeting.

Bivalent chromatin domains at promoters are associated with transcription of ncRNA around few hundred nucleotides of (TSS) transcription start site, which may explain the retention of H3K4me3 mark even on 'poised promoters'. These ncRNAs in turn recruit PRC2 in *cis* for repression state. However certain line of evidence point that TSS ncRNA runs through and dislodges repressive complex and facilitates recruitment of ASH1 (trxG) complex by its direct binding, which further results in release of elongation block for RNA PolII. The PcG/trxG switch needs transcription through corresponding PRE/TRE to overcome silencing (Schmitt et al. 2005). In vertebrates PRE/TRE are not defined, however RA induced transcription of ncRNA through PcG sites has been observed with concomitant loss of SUZ12 and EZH2 from target regions (Sessa et al. 2007) to switch on active state. Like in flies this ncRNA transcription precedes hox expression but ectopically increasing the ncRNA does not have any effect on hox gene expression, suggesting that transcription *per se* is important rather than the stoichiometry.

More than 200 long non-coding RNA (lncRNA) are transcribed through four human hox clusters. One of the lncRNA transcribed from HoxC cluster is 2.2 kb long, named as HOTAIR and targets HoxD cluster (Rinn et al. 2007; Woo and Kingston 2007). Depletion of HOTAIR results in loss of H3K27me3 in HoxD cluster and hence de-repression of many hoxd genes while there is no effect on HoxC genes.

HOTAIR directly binds to PRC2 complex and hence acts as a structural platform to target silencing machinery on HoxD (Tsai et al. 2010). This kind of trans-regulation by ncRNA raises more questions than answers got from ncRNA for cis-regulation where the transcription of ncRNA coincides with its PcG/trxG recruitment. It is not yet known whether recognition of ncRNA by PcG is dependent on primary sequence or on the structural information of ncRNA. Recently a study has shown long non-coding RNAs to be identified having enhancer like activation function (Orom et al. 2010). Some of these long non-coding RNAs have typically H3K4me3 at their start site while others have H3K4me1, both having H3K36 methylation downstream, which is hallmark of transcriptional elongation. Since ectopic expression of these RNAs up-regulates gene expression, it is speculated that TSS of these RNAs can serve as *cis*-enhancers or PRE/TRE domain for the corresponding coding gene.

9.6.7 PcG/trxG Memory

The expression state decided by PcG/trxG is transmitted through DNA replication and cell divisions. The transmission of this epigenetic memory information resides within the chromatin structure itself. That is why several kinds of epigenomes exist in a single animal body with liver cells always giving rise to cells having liver epigenomes and skin cells to cells with skin epigenome after every cell division. The maintenance of cellular memory module is done by PcG/trxG complexes and therefore crucial for differentiation and embryogenesis.

Evidences support the idea that members of PcG/trxG sustain through DNA replication and as a matter of fact their mark do not get 'erased'. PcG bound chromatin even after replication *in vitro* retains binding. trxG proteins like MLL1 and Trl-GAGA have been shown to be associated with mitotic condensed chromosomes and thus enables activation of a wave of transcription quickly upon mitotic exit (Bhat et al. 1996; Blobel et al. 2009). This model summarizes that H3K27me3 mark can also recruit PRC2 during G1 phase in proliferating cells and further methylates daughter strand and adjacent regions to recruit PRC1 silencing complex (Margueron et al. 2009).

One of the mechanisms of PcG silencing is via long-range chromatin looping to maintain transcriptionally poised domains (Bantignies et al. 2011; Lanzuolo et al. 2007). Loss of PcG disrupts this looping and thus leads to de-repression. A recent study shows that the loss of looping can also be due to loss of DNA methyltransferases (DNMT1/3A) (Tiwari et al. 2008). Epigenetically silenced CpG-hypermethylated promoters are known to be overlapping with domain of PcG binding in mammalian genome. It has been shown that promoters having histone H3 lysine 27 methylation are more frequently *de novo* methylated than other promoters (Mohn et al. 2008). On the contrary Dnmt3a dependent non-promoter methylation is shown necessary to counteract PcG silencing and thus maintains the active state of gene expression (Wu et al. 2010). However it is not clear whether DNA methylation that can be transmitted can serve as memory indicator for PcG silencing.

9.7 Epigenomic Constraints and Their Co-Option with Hox Genes for Complexity

Coding regions need appropriate epigenetic environment to perform their functions in a defined cell type. Upgradation in the number of germ layers from single layered poriferans to diploblastic cnidarians to triploblastic organisms is one of the prerequisite mechanisms in specifying cell type specific epigenomes. Increase in number of cell type specific epigenomes is the complexity incorporated in metazoan evolutionary inventory. Lower bilaterians have around 30 cell types, and as the complexity increased during evolution number of cell types also increased from 150 in amphibians to around 200 cell types in mammals irrespective of genome size (Denis and Lacroix 1993; Milinkovitch et al. 2010; Sarras et al. 2002). Certainly, restriction on number of cell types in lower organisms, poses **cell type epigenome constraint** for their evolvability. Increasing cell types effectively increased the number of epigenetic modules not only at the cellular level but also increased the inter-cellular interactions between different cell types. Several cell types have to be networked for a single functional module. For example removal of six somites in vertebrate embryo that give rise to forelimb, leads to abrogation in developing muscular connections and hence defective limb formation (Chevallier et al. 1978; Lee 1992; Lee and Chan 1991).

Many chromatin-mediated factors have duplicated and diverged along with the cell type complexity. Epigenetic regulators of homeotic genes Polycomb/trithorax is one such group that has expanded in vertebrate lineage (Senthilkumar and Mishra 2009) and so have the cell types. Homeotic selector genes specify cell identity not as standalone rather need chromatin reorganization done by the cascade of upstream events. Since in vertebrates, the axis elongation and segment specifications go hand in hand, the role of PcG/trxG in conjunction with homeotic genes is more important. Similarly the members of the family of Wnts, Fgfs, BMPs, RARs (Dale and Jones 1999; Dickinson and McMahon 1992; Hogan 1996; Katsube et al. 2009; Moon et al. 1997; Slack 1990; Yamaguchi and Rossant 1995) and many other important development regulators have increased and diversified in more complex organisms.

9.8 Chromatin Organization of Hox Complex in Retrospect

Primary sequence of DNA imparts information to carry out much of the basic cellular functions, such that ‘naked DNA’ is adequate for basic transcription machinery along with some co-factors to transcribe it into RNA. Only ‘transcription’ of coding region cannot suffice for the sustenance of cellular system that needs higher order of regulated expression of its coding region. The same DNA sequence can project variety of functionalities depending upon how it is packaged, in other words how it is ‘chromatinized’. Such is the profound effect of the chromatin on gene expression that even neighboring state of chromatin matters. Before gene expression to take place it is important to prepare its ‘chromatin state’ and at the same time to ward it

off from the neighboring not so congenial chromatin environment in a most efficient way possible. For example an active gene can be repressed by positioning it near to the inactive chromatin (heterochromatin).

147 bp of linear DNA is wrapped around per nucelosome/histone octamer maintaining at least 14 distinct contact points (Ebralidse et al. 1988; Lambert and Thomas 1986). Despite being dynamic with high turnover, this packaging is energetically most favored and most stable DNA-protein interaction in cellular conditions. The degree of compaction of a nucleosome decorated DNA is a function of histone modifications it carries. There are several post-translational modifications of protruding histone tails that are known to effect the compaction of DNA and hence associated with transcriptionally *active* or *inactive* chromatin marks. Most of these modifications are localized upstream of the coding sequence directly modulating the gene expression. In co-expressed genes histone acetylations (active mark) in one gene are speculated to trigger or spread to the effector genes. However histone methylations are meticulously regulated requiring specific DNA sequence and specific catalytic enzymes resulting in critical structural changes in the chromatin by further recruitment of chromatin complexes (Suganuma and Workman 2008). Acetylated histones relax the DNA compaction and hence facilitate recruitment of co-activators for transcription. The RNA PolII initiation and elongation phase are marked by its Ser-5-CTD and Ser-2-CTD phosphorylation respectively. PAF complex is loaded onto Ser-5 CTD and is central to the recruitment of many Ser-5-CTD associated complexes. PAF is essential for H2B ubiquitination, which is prior to the recruitment of H3K4 di-tri-methyltransferase, Set1 required for elongation (Ng et al. 2003). MLL complex in mammals is essential for converting H3K4me2 into H3K4me3, the mark enriched in the promoter regions of the actively transcribing genes (Wysocka et al. 2005). RNAPolII can run through the nucleosome-DNA complex if at least one H2A/H2B dimer is evicted. ATP dependent remodeling complex RSC, which has multiple bromodomain factors facilitates the eviction of H2A/H2B dimer from the paused RNAPolII sites and FACT histone chaperone complex takes care of the assembly and disassembly of histones during elongation (Belotserkovskaya et al. 2003; Saunders et al. 2003; Workman and Kingston 1998).

Chromatin needs to be removed off the repressive marks and consecutively marked with active histone modifications to come closer to the possibility of being transcribed, as it has been described above that several other factors associated directly with the RNAPolII machinery have to fall in place for productive gene expression. 'Opening up' of HoxD cluster is preceded by the removal of H3K27me3 mark in a temporally collinear fashion and replacement by acetylated histone3 (Soshnikova and Duboule 2009). This temporal opening of HoxD cluster gets disturbed upon gross genomic rearrangement of the hox locus (Fig. 9.3). The de-condensation and opening up of hoxb1 gene upon induction (RA) allows it to 'break free' from its repressive environment to get expressed (Chambeyron and Bickmore 2004a; Chambeyron et al. 2005; Morey et al. 2007, 2008), while hoxb9 is still retained in the inactive region of the chromosomal territory. The looping out of co-expressed genes has been documented in beta-globin clusters also, where LCR induces the looping out of the genes followed by their expression (Chambeyron and Bickmore 2004b). Relocating beta-globin LCR on another chromosome leads

to high expression of genes in the vicinity along with the GFP reporter targeting them to so called RNAPolII speckles or transcription factories (Ragoczy et al. 2006). With the emerging idea of the nucleus being functionally compartmentalized, it has been shown that elongating RNAPolII foci are discrete and distinct than poised RNApoII foci. Within the cluster the looping out of genes is thought to target them to different RNAPolII foci and thus decides the transcription state of the gene (Sutherland and Bickmore 2009).

After induction of hox genes how is chromatin maintained? The answer to this question is still poorly understood. However, 3C based study of human testicular cell lines revealed that resting hox clusters are maintained in distinct loops having contact points within the cluster (Ferraiuolo et al. 2010). Similar looping within the cluster is also observed in tissues expressing beta-globin locus (Tolhuis et al. 2002). Few DNA-FISH studies in *Drosophila* have given an idea that long-range interaction within BX-C is necessary for correct expression of hox genes. Distinct loop interactions were found in different anterior-posterior compartments in the same embryo (Lanzuolo et al. 2007), where expression of hox gene requires it to leave the repressive polycomb bodies. In the head region where both *Ubx* and *Abd-B* repressed, bxd-PRE and Fab7-PRE colocalize with PC body, while in abdomen region they do not. These interactions are abrogated in PC mutations suggesting the requirement of PcG for 'loop maintenance' (Bantignies et al. 2003). Another study reported that insulators of *Mcp* and *Fab7* are required rather than PRE for this long-range interaction in transgenic context (Li et al. 2011).

9.9 Summary and Future Directions

Since past few years understanding of the mechanism of coordinated gene expression of clustered genes is developing in terms of chromatin structure. Emerging scenario for the regulation of homeotic complexes portrays the modular entities as having interdependence for their function. Modular elements like enhancers, boundaries, PRE/TRE, RARE are identified separately in transgenic or deletion assays, but when sewn together, in a typical fashion modulate chromatin in the most desirable manner. It is not necessary that all of these regulatory elements have to be functional along A-P axis, but one's functionality may ensure how the other element would behave in a segment specific manner (Fig. 9.3). The initial opening up of the vertebrate hox cluster is dependent on global mechanism while fine-tuning of the cluster and maintenance may need several modules apposed together. Since vertebrate embryo grows in time and space, the initiation/establishment and maintenance of the complex takes place simultaneously. Contribution of epigenetic mechanisms has emerged as the key factor in this complex regulatory process.

Homeotic gene cluster, specially the one in *Drosophila melanogaster*, has served as an excellent model system for genetic screens and discovery of regulatory mechanisms involved in the genetic basis of animal body plan (Lewis 1978). This and similar, but more extensive genetic screens that followed soon after (Nusslein-Volhard and Wieschaus 1980) have led to the discovery of molecular

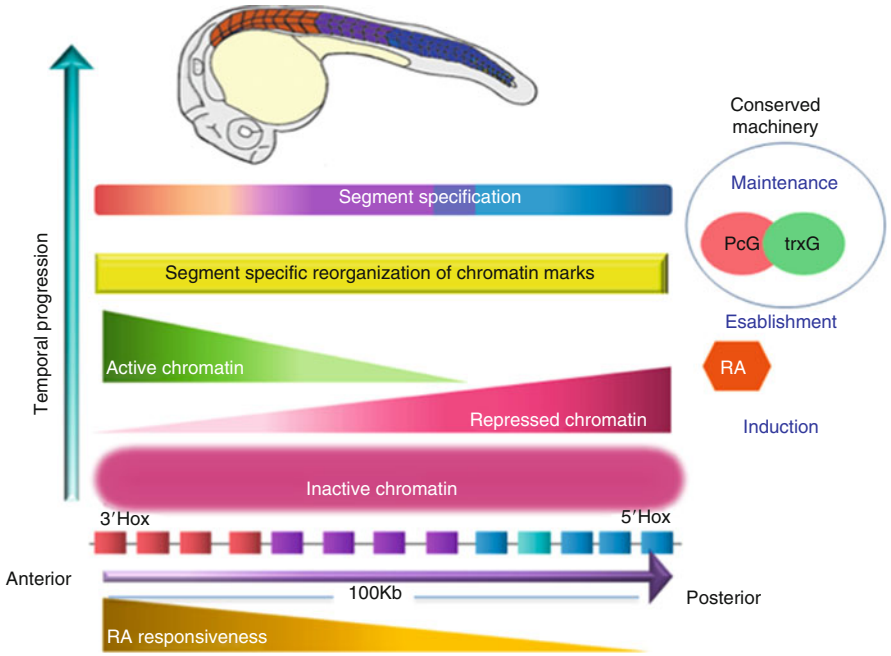

Fig. 9.3 Chromatin organization of hox genes during development. Schematic representation of vertebrate Hox complex is shown. Initially during embryonic stages hox locus is silenced and all the hox genes are in repressed condition. As development proceeds (from *top* to *bottom* of the figure), the genes get expressed one after the other in the order of chromosomal location. 3′ hox genes in the anterior part of the body have active chromatin marks and 5′ hox genes have repressed chromatin. Induction of hox expression is controlled by retinoic acid signaling in vertebrates. Anterior genes are highly responsive or sensitive to retinoic acid induction than posterior genes and hence are expressed earlier even at lower doses of retinoic acid. Once the hox gene expression is induced, it is established and maintained by chromatin reorganization controlled by PcG/trxG proteins. The segment specific reorganization of chromatin marks determines the correct body patterning along A-P axis

components of embryonic development that have turned out to be amazingly conserved from flies to human. These historic screens and analyses paved the way to understand the molecular basis of embryonic development and led us to the point where the question that stares at us is – how the identical genome gives hundreds of cell type during embryonic development? Can we have 'epigenetic screens' to dissect out epigenetic details? One needs a way to isolate factors in cell type and temporally specified context as against the genetic screens that lead to the final phenotypes in an organism. While this appears to be an intractable problem, one way forward is to map the epigenetic profile of a cell type, in a temporally defined context. This and a 3D organization of the genome within the nuclear space of specific cells in a temporal context can give us the ultimate set of information to understand how the single genome gives multiple cell types.

Epigenetic mechanisms, by definition, act differentially on the identical genome in distinct cell types. This also means that cis-elements that recruit or guide epigenetic modifications of specific loci are interpreted differently by recruitment factors in different cell types. Identification of such 'Epi-cis-elements' of the genome will be a step forward to understand how this regulation of highest dimension and dynamics has evolved to take genetic information to the level of sophistication and complexity as is seen in organisms of such a vast variety.

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Part III
Epigenetics and Transcription Regulation

Chapter 10
Basic Mechanisms in RNA Polymerase I Transcription of the Ribosomal RNA Genes

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Abstract RNA Polymerase (Pol) I produces ribosomal (r)RNA, an essential component of the cellular protein synthetic machinery that drives cell growth, underlying many fundamental cellular processes. Extensive research into the mechanisms governing transcription by Pol I has revealed an intricate set of control mechanisms impinging upon rRNA production. Pol I-specific transcription factors guide Pol I to the rDNA promoter and contribute to multiple rounds of transcription initiation, promoter escape, elongation and termination. In addition, many accessory factors are now known to assist at each stage of this transcription cycle, some of which allow the integration of transcriptional activity with metabolic demands. The organisation and accessibility of rDNA chromatin also impinge upon Pol I output, and complex mechanisms ensure the appropriate maintenance of the epigenetic state of the nucleolar genome and its effective transcription by Pol I. The following review presents our current understanding of the components of the Pol I transcription machinery, their functions and regulation by associated factors, and the mechanisms operating to ensure the proper transcription of rDNA chromatin. The importance of such stringent control is demonstrated by the fact that deregulated Pol I transcription is a feature of cancer and other disorders characterised by abnormal translational capacity.

10.1 Introduction

In eukaryotic cells, the task of transcribing nuclear genes is shared by Pols I, II and III. Each of these polymerases is dedicated to the transcription of a different set of genes, known as class I, II or III genes, accordingly. Pol II produces messenger (m) RNAs, which code for cellular proteins, and many small nuclear (sn)RNAs, which

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are involved in mRNA processing. Pol III synthesises the transfer (t)RNAs, the 5S rRNA and a variety of other small, untranslated RNAs with essential roles in metabolism (White 2008). Unlike Pols II and III, which transcribe a variety of different genes, Pol I is dedicated to the synthesis of rRNA, and this accounts for up to 60% of transcriptional activity in a eukaryotic cell (Moss and Stefanovsky 2002). In mammals, the 47S pre-rRNA produced by Pol I is processed into mature 18S, 5.8S and 28S rRNAs, which are essential structural and catalytic components of ribosomes (Moore and Steitz 2002). Ribosomes constitute the core of the protein synthetic machinery; consequently, ribosome content is a critical determinant of protein accumulation and hence cell growth and division (Camacho et al. 1990; Siehl et al. 1985; Zetterberg and Killander 1965). The abundance of ribosomes within a cell depends upon the availability of rRNA (Liebhaber et al. 1978). Therefore, given the high metabolic burden of rRNA production and its direct influence on protein synthetic capacity, a tightly regulated transcription system has evolved, devoted to ensuring that rRNA synthesis is closely coupled with cellular growth demands.

10.2 The Ribosomal DNA

Ribosomal RNA synthesis by Pol I occurs in the nucleolus. This nuclear structure is also the site of ribosome assembly, which involves the incorporation of the rRNAs produced by Pol I along with Pol III-produced 5S rRNA and many ribosomal proteins (for reviews see Boisvert et al. 2007; Tschochner and Hurt 2003). The nucleolus forms around the nucleolar organiser regions (NORs) containing hundreds of rRNA genes, the majority (although not all) of which are organised head-to-tail into tandem arrays (Caburet et al. 2005; Nemeth and Langst 2011). Recent genome-wide mapping studies aimed at characterising the nucleolar genome have discovered that specific chromatin regions unrelated to the rDNA, and localised to distinct chromosomes, are also associated with nucleoli (Nemeth et al. 2010; van Koningsbruggen et al. 2010). These studies point towards a role for the nucleolus in the general organisation of chromosomes in the nucleus and imply a correlation between tethering to the nucleolar periphery and transcriptional silencing.

Approximately 150–200 rDNA repeats are found in the yeast genome, whereas human diploid cells have approximately 400 repeats (Birch and Zomerdijk 2008). However, only a subset of these genes (~50%) is transcribed at any given time. A recent study proposed that allelic inactivation of mammalian rRNA genes, occurring early in development, could account for this (Schlesinger et al. 2009). Active and inactive rDNA repeats are distinguished by distinct chromatin states, epigenetic marks, topological organisation and sub-nucleolar localisation (for reviews see Birch and Zomerdijk 2008; McStay and Grummt 2008; Nemeth and Langst 2011; Sanij and Hannan 2008). Transcriptionally inactive rDNA is found in a tightly packaged, heterochromatic state characterised by methylation of the DNA and repressive histone modifications, and is localised outwith the nucleolar fibrillar centre/fibrillar centre-dense fibrillar component border regions where rDNA transcription takes

place. On the other hand, active rRNA genes are found in a more open chromatin state characterised by hypomethylated DNA, acetylated histones and, in mammals, an enrichment of the Pol I activator upstream binding factor (UBF). A related protein, Hmo1, specifically associates with active rDNA repeats in yeast (Merz et al. 2008). UBF binds DNA as a dimer through its high mobility group (HMG) domains, inducing substantial topological changes in DNA (Bazett-Jones et al. 1994; Jantzen et al. 1990). UBF binding is thought to be important for maintaining euchromatic rDNA, partly through its displacement of the repressive histone H1 (Kermekchiev et al. 1997; Nemeth and Langst 2011; Sanij et al. 2008) and is critical for nucleolar architecture, underpinning the structural organisation of rDNA into NORs (Mais et al. 2005). UBF has recently been shown to interact with the transcriptional regulator CTCF (CCCTC binding factor) (van de Nobelen et al. 2010), which has been implicated in the regional organisation of nucleolar rDNA (Guerrero and Maggert 2011). Extensive DNA looping is postulated to occur specifically in the active rDNA repeats, juxtaposing sites of transcription initiation and termination, mediated by Pol I-specific transcription factors and the proto-oncogene c-Myc (Denissov et al. 2011; Nemeth et al. 2008; Shiue et al. 2009). This higher order chromatin conformation of active rRNA genes further demonstrates the complexity of genome organisation within the nucleolus, the regulation of which is only just beginning to be understood.

In mammals, each rDNA repeat is approximately 43 kb and contains regulatory elements including promoters, repetitive enhancers and terminators within an intergenic spacer (IGS) of approximately 30 kb, and a single transcribed region of approximately 13 kb containing the 47S coding region (Fig. 10.1) (reviewed by McStay and Grummt 2008). In *S. cerevisiae*, each rDNA repeat is approximately 9.1 kb and contains a 6.9 kb 35S pre-rRNA coding region and a comparatively short IGS (Albert et al. 2011; French et al. 2003). Although described as rDNA 'repeats', recent evidence indicates that the multiple rRNA genes are not simply identical copies of the same transcription unit but that, in fact, several rDNA variants exist and these can be differentially expressed and regulated (Santoro et al. 2010; Tseng et al. 2008).

Eukaryotic rRNA gene promoters contain two regulatory elements important for directing accurate and efficient transcription initiation: the core promoter and the upstream control element (UCE; functionally analogous to the yeast upstream promoter element (UPE)) (Fig. 10.1). The core promoter is sufficient for basal transcription by Pol I in most species (Paule and White 2000). The UCE lies further upstream (−156 to −107 relative to the transcription start site of human rRNA genes) and is important for stimulating transcription from the core promoter (Paule and White 2000; Russell and Zomerdijk 2005). Although the general layout of the rDNA promoter is conserved from yeast to humans, with the spacing and orientation of the core promoter and UPE/UCE being critical, there is little sequence similarity between elements and, as a result, Pol I transcription is highly species specific (for reviews see Grummt 2003; Heix and Grummt 1995). In addition to the main rRNA gene promoter, which directs pre-rRNA synthesis, related sequence elements known as spacer promoters have been identified within the IGS from several species

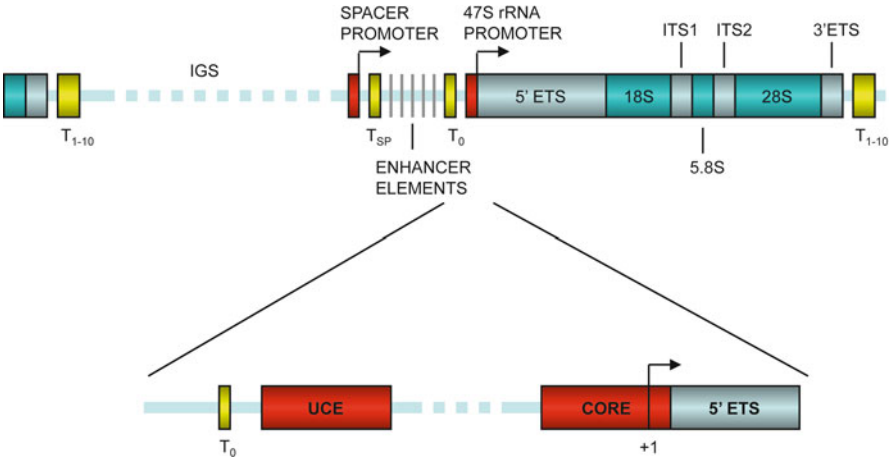

Fig. 10.1 Mammalian rDNA repeat and 47S rRNA promoter. The *top* panel illustrates key elements and the general organisation of a mammalian rDNA repeat. The IGS includes the spacer and 47S rRNA promoters, enhancer repeats and the TTF-I binding sites T_0 and T_{sp}. *Arrows* indicate start sites and direction of transcription. The coding region contains 5′ and 3′ external transcribed spacer (ETS) and two internal transcribed spacer (ITS) regions, along with regions encoding 18S, 5.8S and 28S rRNAs. Terminator elements ($T_{1\text{-}10}$) downstream of the 47S rRNA gene are also indicated. The *lower* panel illustrates the layout of the 47S rRNA promoter, which directs the assembly of the Pol I PIC and consists of an upstream control element, and a core promoter element overlapping the transcription start site

(De Winter and Moss 1986; Grimaldi and Di Nocera 1988; Kuhn and Grummt 1987; Labhart and Reeder 1984) (Fig. 10.1). Studies using mouse cells suggest that transcripts produced by Pol I from these promoters (known as promoter RNA (pRNA)) are involved in transcriptional silencing of the rRNA genes (Mayer et al. 2006).

In plants, IGS transcripts of as yet undefined origin serve as precursors for the RNA-dependent RNA Polymerase 2 (RDR2). RDR2 works together with, among other enzymes, the plant-specific Pols IV and V in the production of siRNAs which also mediate class I gene silencing through epigenetic mechanisms (Lawrence et al. 2004; Pontes et al. 2006; Preuss et al. 2008).

10.3 Transcription by Pol I

Using the rDNA repeats as a template, Pol I catalyses the synthesis of rRNA. However, in order to do this accurately and efficiently, Pol I requires a number of accessory factors, which facilitate polymerase recruitment, initiation, promoter escape, elongation, termination and re-initiation, as discussed below.

10.3.1 Pre-initiation Complex (PIC) Assembly

RNA polymerases themselves have little affinity for promoter sequence elements and so rely upon specific transcription factors for accurate recruitment. Therefore, as with Pol II- and Pol III-driven transcription, the first stage of transcription by Pol I is the formation of a PIC at the gene promoter. A common feature of the basal transcription machinery used by Pols I, II and III is the requirement for a TBP-containing transcription factor complex. However, in each case, the combination of TBP-associated factors (TAFs) is polymerase-specific. Transcription by Pol I in mammalian cells is dependent upon selectivity factor 1 (SL1, termed TIF1B in mouse), which is a complex of TBP and at least four Pol I-specific TAFs: TAF_I110 (TAF1C), TAF_I63 (TAF1B), TAF_I48 (TAF1A) and TAF_I41 (TAF1D) (Comai et al. 1992, 1994; Eberhard et al. 1993; Gorski et al. 2007; Heix et al. 1997; Zomerdijk et al. 1994). An additional TAF, TAF12, which was originally described as a factor involved in the transcription of class II genes, has also been implicated as a component of the mammalian SL1 complex (Denissov et al. 2007).

SL1 is pivotal to PIC formation. It confers promoter specificity by recognising and binding the core promoter element in the rDNA repeat, it is essential for Pol I recruitment to the transcription start site, and it promotes a stable interaction between UBF and the rDNA promoter (Beckmann et al. 1995; Cavanaugh et al. 2002; Friedrich et al. 2005; Miller et al. 2001; Rudloff et al. 1994). Recently, SL1 has been shown to also have an essential post-polymerase recruitment role, operating through TAF1B (Naidu et al. 2011). TAF1B and the yeast orthologue Rrn7 are structurally and functionally related to TFIIB and the Brf proteins, which are involved in Pol II and Pol III transcription, respectively, thus extending and underscoring the parallels between the eukaryotic transcription machineries (Knutson and Hahn 2011; Naidu et al. 2011).

Furthermore, a role for SL1 in maintaining the promoters of active rRNA genes in a hypomethylated state has been proposed. This involves the TAF12-mediated recruitment of GADD45a (growth arrest and DNA damage inducible protein 45 alpha) and various components of the nucleotide excision repair (NER) machinery (Schmitz et al. 2009). In addition, SL1 is thought to contribute to the structural organisation of actively transcribed rRNA genes through interactions with promoter, upstream enhancer and terminator elements (Denissov et al. 2011). Transcription by Pol I in yeast also requires TBP and the core promoter binding complex core factor (CF), which is composed of three associated proteins, RRN6, 7 and 11 (Reeder 1999). RRN6, 7 and 11 are only distantly related to the mammalian SL1 subunits TAF_I110, TAF_I63 and TAF_I48, respectively, with limited sequence homologies (Boukhgalter et al. 2002; unpublished observations), highlighting the divergent nature of the rDNA promoter elements.

Core promoter selection and binding by SL1 is solely mediated by the TAFs, with TAF_I110, TAF_I63 and TAF_I48 being reported to make direct contacts with the DNA (Beckmann et al. 1995; Rudloff et al. 1994). SL1 TAFs are also crucial for the recruitment of Pol I. Pol I has 14 polypeptide subunits in yeast, homologues for 13

Table 10.1 Eukaryotic RNA polymerase subunits

S. cerevisiae Pol I subunits	Human Pol I subunits	Homologues in Pols II/III [or associated factors]
Shared subunits		
RPB5 (ABC27, POLR2E)	hRPB5	shared
RPB6 (ABC23, POLR2F)	hRPB6	shared
RPB8 (ABC14.5, POLR2H)	hRPB8	shared
RPB10 (ABC10β, POLR2L)	hRPB10	shared
RPB12 (ABC10α, POLR2K)	hRPB12	shared
RPA40 (AC40, POLR1C)	hRPA40	RPB3/shared
RPA19 (AC19, POLR1D)	hRPA19	RPB11/shared
Homologous subunits		
RPA190 (A190, POLR1A)	hRPA190	RPB1/RPC160
RPA135 (A135, POLR1B)	hRPA135	RPB2/RPC128
RPA43 (A43, POLR1F)	hRPA43	RPB7/RPC25
RPA14 (A14)	*	RPB4/RPC17
RPA12 (A12.2, POLR1H)	hRPA12	RPB9/RPC11
RPA49 (A49, POLR1E)	PAF53	[TFIIF (Rap74 subunit) & TFIIE-β/RPC37 & RPC34]
RPA34.5 (A34.5, POLR1G)	CAST (PAF49)	[TFIIF (Rap30 subunit)/ RPC53]

*Human counterpart not yet identified

of which have been identified in mammals (Table 10.1). A catalytic core is formed by ten of these subunits, which are shared with or homologous to subunits found in Pols II and III (Kuhn et al. 2007; Werner et al. 2009). At the periphery, the A14 and A43 Pol I subunits associate as a heterodimer (Kuhn et al. 2007). Similar heterodimeric structures are also found in Pols II and III (see Table 10.1 for homologous subunits) (Werner et al. 2009). The remaining two Pol I-specific subunits (*S. cerevisiae* A49 and A34.5; mammalian PAF53 and CAST/PAF49) form a heterodimeric subcomplex that can dissociate from Pol I (Hanada et al. 1996; Huet et al. 1975; Kuhn et al. 2007; Yamamoto et al. 2004). Studies in yeast indicate that this subcomplex is structurally and functionally related to the TFIIE and TFIIF initiation factors used by Pol II (as indicated in Table 10.1), and can bind DNA and promote RNA cleavage (Geiger et al. 2010; Kuhn et al. 2007). These specific Pol I subunits function at multiple stages in the Pol I transcription cycle, playing important roles in polymerase recruitment, promoter escape and elongation (Albert et al. 2011; Beckouet et al. 2008; Kuhn et al. 2007; Panov et al. 2006a, b).

The multisubunit Pol I complex exists as at least two distinct subpopulations (Milkereit and Tschochner 1998; Miller et al. 2001), known as Pol Iα and Pol Iβ in mammalian cells (Miller et al. 2001). Both forms of Pol I are active and can catalyse the synthesis of RNA, but only Pol Iβ, which represents less than 10% of the total Pol I in a cell, can be incorporated into PICs and initiate accurate, promoter-specific

transcription (Milkereit and Tschochner 1998; Miller et al. 2001). This is due, at least in part, to the association of Pol Iβ with RRN3 (Milkereit and Tschochner 1998; Miller et al. 2001) (murine TIF1A (Bodem et al. 2000)). RRN3 interacts directly with Pol I, through its A43 subunit (Cavanaugh et al. 2002; Peyroche et al. 2000). In addition, the Pol I-specific A49/A34.5 subcomplex is important for the association of RRN3 with Pol I, although it is unclear whether this is mediated by direct interactions between RRN3 and these subunits (Beckouet et al. 2008). RRN3 also binds the CF subunit RRN6 in yeast, and SL1 subunits TAF_I110, TAF_I63 and TAF_I41 in mammals (Cavanaugh et al. 2002; Gorski et al. 2007; Miller et al. 2001; Peyroche et al. 2000). Therefore, RRN3 plays an essential, evolutionarily-conserved role in mediating specific transcription initiation at class I genes by connecting Pol I with an essential promoter-binding factor, and thus facilitating polymerase recruitment to the PIC at the rDNA promoter.

In addition to RRN3, various other proteins have been found specifically associated with Pol Iβ. For example, the serine/threonine kinase CK2 is present in Pol Iβ but not Pol Iα complexes and is found at the rDNA promoter in cells (Lin et al. 2006; Panova et al. 2006). Various roles have been proposed for this kinase in the regulation of transcription by Pol I (Bierhoff et al. 2008; Lin et al. 2006; Panova et al. 2006; Voit et al. 1992). Reports suggest that CK2 targets TAF_I110 and UBF and in this way regulates PIC assembly and stability, although the precise mechanistic details of this remain unclear (Lin et al. 2006; Panova et al. 2006). CK2 also phosphorylates the essential initiation factor TIF1A (the mouse counterpart of RRN3) (Bierhoff et al. 2008). However, rather than influencing PIC assembly, this modification seems important for the release of Pol I from promoter-bound initiation factors and thus elongation (Bierhoff et al. 2008). Another protein found specifically associated with the initiation-competent Pol Iβ complex is topoisomerase IIα (Panova et al. 2006). Interestingly, this topoisomerase IIα was found to be targeted by Pol Iβ-associated CK2. However, the significance of these observations to the regulation of transcription by Pol I have yet to be elucidated.

SL1 and Pol Iβ alone are sufficient to support basal levels of Pol I transcription *in vitro*. However, to achieve activated transcription, UBF must also be incorporated into the Pol I PIC. As discussed above, UBF binds throughout the rDNA in cells (O'Sullivan et al. 2002), playing critical roles as a nucleolar scaffold protein and in promoting decondensation of rDNA chromatin (Chen et al. 2004; Mais et al. 2005; Sanij et al. 2008). Crucially, UBF can also activate promoter-specific transcription by Pol I. UBF interacts cooperatively with SL1 at the rDNA promoter, with SL1 binding the highly acidic C-terminus of UBF through its TAF_I48 and TBP subunits (Beckmann et al. 1995; Bell et al. 1988; Hempel et al. 1996; Jantzen et al. 1992; Kihm et al. 1998; Tuan et al. 1999). This stabilises the association of UBF with the PIC (Friedrich et al. 2005), hence facilitating promoter-specific transcriptional activation. In addition to SL1, UBF also interacts with the PAF53 and PAF49/CAST subunits of Pol I (Hanada et al. 1996; Panov et al. 2006a, b; Seither et al. 1997; Whitehead et al. 1997). The HMG-box protein Hmo1 is involved in rDNA transcription in yeast and is, perhaps, the functional analogue of mammalian UBF (Gadal et al. 2002). Like UBF, Hmo1 binds throughout the rDNA repeat and acts synergistically

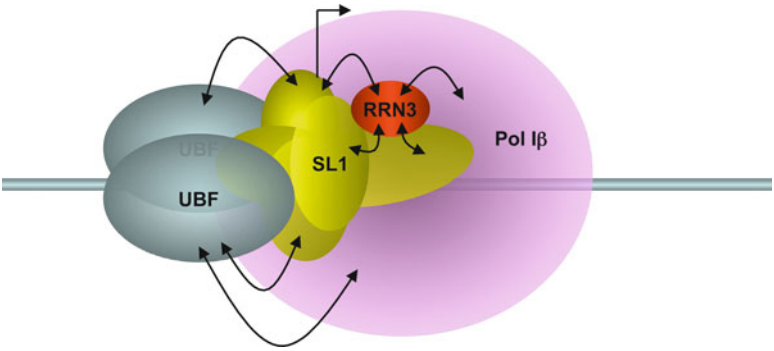

Fig. 10.2 The mammalian Pol I pre-initiation complex. Activated transcription by Pol I requires the assembly of Pol I-specific transcription factors SL1 and UBF at the rRNA promoter. In addition to contacts made between these transcription factors and the rDNA, several protein-protein interactions are also known to facilitate PIC assembly, as indicated by double-headed *arrows* (described in the text). A multitude of other factors cooperate with this transcription machinery to enhance PIC assembly and promote efficient rRNA synthesis by Pol I *in vivo*

with the Pol I-subunit A49 to activate transcription (Gadal et al. 2002; Hall et al. 2006; Kasahara et al. 2007). Interestingly, Albert et al. (2011) recently demonstrated the importance of the yeast A34.5-A49 subcomplex to nucleolar architecture. Given the fundamental role played by UBF as a nucleolar scaffold, it will be interesting to ascertain whether this property of these Pol I subunits is influenced by their interaction with Hmo1/UBF.

In summary, a series of cooperative protein-protein and protein-DNA interactions involving SL1, UBF (or their functional equivalents) and specific promoter elements are required for the recruitment of polymerase poised for the activated transcription of rRNA genes, as depicted in Fig. 10.2. Live cell imaging coupled with computational kinetic modelling has demonstrated a direct correlation between the efficiency of PIC assembly and transcriptional output in cells (Gorski et al. 2008).

10.3.2 Initiation and Promoter Escape

Following the assembly of a productive PIC at the rDNA promoter, promoter opening and transcription initiation by Pol I can commence, defined by the incorporation of the first ribonucleotides of the RNA chain. However, for productive RNA synthesis to ensue, Pol I must dissociate from the promoter-bound initiation factors in a process known as promoter escape (Panov et al. 2006a, b; Russell and Zomerdijk 2005). This post-PIC assembly event is rate-limiting for rRNA synthesis *in vitro* (Panov et al. 2001).

Promoter escape following transcription initiation coincides with the release of RRN3 from polymerase (Aprikian et al. 2001; Hirschler-Laszkiewicz et al. 2003; Milkereit and Tschochner 1998). In mouse cells, covalent attachment of RRN3 to the A43 Pol I subunit, with which RRN3 interacts, impairs rDNA transcription and cell cycle progression (Bierhoff et al. 2008). However, a similar approach pioneered in yeast strains lacking RRN3 and A43, but instead expressing a non-dissociable Pol I-RRN3 complex, did not detect any defects in rRNA synthesis or growth (Laferte et al. 2006). These studies suggest potential species-specific differences in the relative importance of RRN3 dissociation to the transcription cycle. The interaction between RRN3 and Pol I is controlled at least in part by phosphorylation. However, regulatory phosphorylation events also appear to vary from yeast to mammals: in yeast, Pol I phosphorylation apparently regulates this interaction, whereas in mammals, RRN3 phosphorylation seems important (Bierhoff et al. 2008; Cavanaugh et al. 2002; Fath et al. 2001). Bierhoff et al. (2008) looked specifically at the phosphorylation events regulating the dissociation of Pol I from RRN3 during promoter escape, and demonstrated that phosphorylation of two specific serine residues in mouse RRN3 (TIF1A) by CK2 promotes its release from polymerase.

The mammalian activator of Pol I transcription, UBF, also plays an important role in stimulating promoter escape (Panov et al. 2006a). UBF interacts with the Pol I-specific heterodimer PAF49/CAST-PAF53 (Hanada et al. 1996; Panov et al. 2006b), and this is important for transcriptional activation by UBF, which occurs subsequent to PIC assembly (Panov et al. 2006b). However, the mechanisms underlying this are unclear, although changes in DNA and/or polymerase conformation have been proposed (Panov et al. 2006b). In yeast, the homologues of these Pol I subunits (A34.5-A49) interact structurally and functionally with the probable yeast counterpart of UBF, Hmo1, and play an important role in promoter escape by promoting the release of RRN3 from elongating polymerase (Beckouet et al. 2008; Gadal et al. 2002; Schnapp et al. 1994). Conceivably, a network of interactions involving these factors could induce conformational changes in the PIC, triggering any post-translational modifications and the release of RRN3, converting initiation-competent Pol I into an elongating form.

10.3.3 Elongation

A mammalian cell requires approximately 8–10 million rRNA transcripts every 24 h to sustain adequate levels of ribosome biogenesis (Lewis and Tollervey 2000). Accordingly, transcription elongation by Pol I is highly efficient with, on average, 100 polymerases transcribing each active gene at a rate of approximately 95 nucleotides per second (Dundr et al. 2002). Similar elongation rates have also been observed for yeast Pol I (French et al. 2003). This impressive transcriptional output is achieved through the intrinsic processivity of Pol I and its cooperation with a multitude of other proteins.

Factors TFIIF and TFIIS are involved in elongation by Pol II (Saunders et al. 2006). Recent work using yeast has demonstrated that the A34.5-A49 subcomplex of Pol I is structurally and functionally analogous to TFIIF and is important for Pol I processivity (Geiger et al. 2010; Kuhn et al. 2007). Furthermore, the A12.2 subunit of Pol I stimulates the intrinsic RNA cleavage activity of Pol I and shares functional and structural homology with TFIIS, which enhances the weak 3′-RNA cleavage activity of Pol II (Haag and Pikaard 2007; Kuhn et al. 2007). This cleavage activity might be required for RNA proofreading and to stimulate elongation by creating a new and correctly aligned 3′OH in the polymerase active site after stalling and backtracking of polymerase. A role for the A34.5-A49 subcomplex in permitting contact between adjacent Pol I molecules on the same rDNA template, which might contribute to efficient transcription elongation, has also been proposed (Albert et al. 2011).

Pol I-specific transcription factors are also thought to play a role in elongation. UBF is distributed throughout the rDNA repeats and has been reported to regulate Pol I elongation by phosphorylation-dependent remodelling of the rDNA chromatin (O'Sullivan et al. 2002; Stefanovsky et al. 2006). More recently, SL1 has been proposed to assist elongating Pol I via its role in anchoring the core promoter, upstream region and terminator, which provides a spatial arrangement favourable for productive rRNA synthesis (Denissov et al. 2011).

Furthermore, several additional factors are crucial for transcription elongation by Pol I in cells, allowing the polymerase to negotiate rDNA in the context of chromatin. For example, the histone chaperones nucleolin, nucleophosmin and FACT assist in rDNA transcription in mammalian cells (Birch et al. 2009; Murano et al. 2008; Rickards et al. 2007) and in yeast, Spt4/5 and Paf1C are important (Schneider et al. 2006, 2007; Zhang et al. 2009, 2010). Moreover, various chromatin remodelling and modifying activities have been shown to promote transcription by Pol I *in vivo* including Chd1p, Isw1p and Isw2p in yeast (Jones et al. 2007) and tip60, Williams syndrome transcription Factor (WSTF)-SNF2h and the histone methyltransferase G9a in mammalian cells (Halkidou et al. 2004; Percipalle et al. 2006; Yuan et al. 2007). Nuclear actin and myosin I drive transcription elongation by Pol I and this might, in part, be attributed to their interaction with the chromatin remodeler WSTF at the rDNA (Percipalle et al. 2006; Ye et al. 2008).

Given the high loading density of Pol I on rRNA genes, it is important that any other physical impediments encountered by a transcribing polymerase, caused by topological changes in the rDNA or DNA damage, for example, are efficiently resolved. Consequently, mechanisms ensure that such constraints are minimised. For instance, topoisomerases promote transcriptional elongation in yeast by relieving the positive and negative supercoiling that occurs ahead of and behind transcribing Pol I, respectively (Brill et al. 1987; El Hage et al. 2010; French et al. 2011; Schultz et al. 1992). Topoisomerase IIα is a component of Pol Iβ in human cells, as discussed above (Panova et al. 2006). Furthermore, topoisomerase I was found associated with Pol I complexes in mouse cells (Hannan et al. 1999; Rose et al. 1988), and has been proposed to assist transcriptional elongation by Pol I in human cells (Zhang et al. 1988). Therefore, this function of topoisomerases in relieving torsional strain during transcriptional elongation by Pol I may be evolutionarily conserved.

Signalling pathways invoked by DNA damage lead to a transient repression of rRNA synthesis, partly through the ATM-mediated displacement of elongating polymerase (Kruhlak et al. 2007). Resumption of Pol I transcription is dependent upon functional DNA repair mechanisms (Kruhlak et al. 2007). Transcription-coupled DNA repair occurs at rDNA genes (Conconi et al. 2002) and various DNA repair proteins have been found in Pol I complexes, including TFIIH, Cockayne syndrome B protein (CSB), Werner's syndrome helicase (WRN), Ku70/80 and several components of the NER machinery (Bradsher et al. 2002; Hannan et al. 1999; Iben et al. 2002; Schmitz et al. 2009; Shiratori et al. 2002). In many cases, these interactions have been shown to promote transcription by Pol I. However, a direct role for these factors in the transcription-coupled repair of rDNA has yet to be demonstrated.

As elongation by Pol I proceeds, the nascent pre-rRNA associates with components of the processing machinery, allowing co-transcriptional maturation of the rRNA and assembly of ribosomal particles (reviewed by Granneman and Baserga 2005). As a result, pre-rRNA synthesis and processing are closely coordinated, such that defective transcription by Pol I impairs pre-rRNA processing and vice versa (Granneman and Baserga 2005; Schneider et al. 2006, 2007). Although the mechanisms responsible for this coupling are incompletely defined, factors implicated in yeast include Spt4 and Spt5, which interact both with elongating Pol I and components of the pre-rRNA processing machinery (Leporé and Lafontaine 2011; Schneider et al. 2006, 2007). Such rigorous coordination likely contributes to the highly efficient and tightly regulated production of ribosomes.

10.3.4 Termination

Transcription termination by Pol I is a multistep process involving specific DNA sequence elements and regulatory proteins. In mammals, transcription termination factor TTF-I binds terminator elements downstream of the rRNA gene (T_1-T_{10}; Fig. 10.1), causing polymerase pausing. Dissociation of the paused transcription complex is then mediated by Pol I and transcript release factor PTRF. A similar mechanism is thought to operate in yeast, involving the TTF-I homologue Reb1p (Jansa and Grummt 1999). However, recent studies using yeast have uncovered further complexity in the control of transcription termination by Pol I, by demonstrating the existence of a 'torpedo' mechanism, similar to that employed for the termination of transcription by Pol II. This process begins with cleavage of the nascent pre-rRNA by the endonuclease Rnt1, followed by the progressive digestion of the resulting Pol I-associated RNA cleavage product mediated by the cooperative actions of the 5′ to 3′ exonuclease Rat1 (mammalian Xrn2) and the RNA helicase Sen1 (Braglia et al. 2010, 2011; El Hage et al. 2008; Kawauchi et al. 2008). Recognition of the Rnt1-cleaved pre-rRNA by Rat1 is thought to be controlled by phosphorylation of the 5′end of the RNA by the polynucleotide kinase Grc3 (Braglia et al. 2010). Once Rat1 reaches elongating Pol I, the transcription complex becomes unstable and dissociates from DNA, thus resulting in transcription termination.

In addition, the smallest Pol I subunit A12.2/RPA12 is critical for effective transcription termination, potentially mediated by its stimulation of the intrinsic 3′-end RNA cleavage activity of Pol I (Haag and Pikaard 2007; Kuhn et al. 2007; Prescott et al. 2004). The Pol II and III homologues of RPA12, RPB9 and RPC11, are also important for relief of polymerase pausing and termination. Furthermore, each of these polymerase subunits shares homology with the RNA cleavage enhancing factor TFIIS (Prescott et al. 2004). It is possible that several mechanisms co-exist to ensure accurate, efficient termination of transcription by Pol I and thus cell viability (Braglia et al. 2011).

10.3.5 Re-initiation

Correct termination of transcription and release of the nascent rRNA is required for re-initiation by Pol I. Once a gene is activated, the rate of re-initiation will contribute to the overall level of transcripts produced. In addition to terminator elements located downstream of the rRNA coding region, TTF-I binding sites are also found immediately upstream of the rDNA promoter (termed T_0) and downstream of the spacer promoter (T_{sp}) (Fig. 10.1) (Nemeth et al. 2008). Interactions between TTF-1 and its binding sites are thought to be important for epigenetic and topological regulation of the rDNA (for reviews see McStay and Grummt 2008; Nemeth and Langst 2011). In cells, efficient recycling and re-initiation by Pol I might be facilitated by TTF-1-mediated juxtaposition of the terminator and promoter elements, which results in the formation of DNA loops (Nemeth et al. 2008; Nemeth and Langst 2011; Shiue et al. 2009). In addition to TTF-I, SL1 and c-Myc have been implicated in the formation of such DNA loops (Denissov et al. 2011; Nemeth and Langst 2011; Shiue et al. 2009).

Another important event in transcription re-initiation by Pol I is the re-association of the essential initiation factor RRN3 with polymerase, allowing Pol I to re-assemble with SL1 and UBF, which remain promoter bound following escape of elongating polymerase (Lin et al. 2006; Panov et al. 2001). This reversible interaction between Pol I and RRN3 underpins critical transitions in the Pol I transcription cycle, and is evolutionarily conserved. However, the mechanisms underlying this are not fully elucidated. Such a dynamic partnership is likely controlled through reversible post-translational modifications (Bierhoff et al. 2008; Cavanaugh et al. 2002; Fath et al. 2001). In support of this, Bierhoff et al. (2008) demonstrated that dephosphorylation of the CK2 target sites (serines 170 and 172) in TIF-1A (mouse RRN3) by the protein phosphatase FCP1 is required for the re-association of TIF-1A with Pol I, and hence re-initiation. In contrast, these particular residues are not conserved in yeast RRN3 and phosphorylation of Pol I itself is thought to regulate Pol I-RRN3 complex formation (Bierhoff et al. 2008; Fath et al. 2001). FCP1 has been implicated in the regulation of Pol I transcription in yeast, but rather than functioning in re-initiation, this phosphatase appears to promote chain elongation (Fath et al. 2004). Whether additional kinases and/or phosphatases targeting RRN3 and/or Pol I are involved in a conserved mechanism for promoting multiple rounds of transcription remains to be determined.

10.4 Regulation of rDNA Transcription

Stringent regulatory mechanisms operate to ensure a precise balance between the requirement for and availability of rRNA, allowing cells to control their capacity for protein synthesis in response to changing metabolic needs. For example, transcription by Pol I is low when nutrients or mitogens are limiting, but upregulated when the availability of these growth stimuli increase. In addition, Pol I transcription is regulated in response to a range of cellular stresses and during many fundamental cellular processes, including cellular differentiation and throughout the cell cycle (for reviews see Drygin et al. 2010; Grummt 2003; Grummt and Voit 2010; Mayer and Grummt 2005; Moss 2004; Russell and Zomerdijk 2005). The level of cellular rRNA is determined by the rate at which active rRNA genes are transcribed, and also by the number of active genes. Some of the mechanisms influencing these different aspects of class I gene expression are discussed below.

10.4.1 Regulation of the Pol I Transcription Machinery

In yeast, growth-dependent changes in rRNA synthesis can be achieved both through altering the proportion of active genes, and the rate of transcription from already active loci (Grummt and Pikaard 2003; Russell and Zomerdijk 2005). However, in mammalian cells, changes in rRNA production in response to growth signals seem to be mediated mainly by the latter mechanism. A plethora of cellular control pathways have been shown to mediate such acute changes in rRNA synthesis by directly regulating the activity of the Pol I transcription machinery, with positive regulators of growth activating transcription and negative regulators of growth having repressive effects (Drygin et al. 2010; Grummt 2003; Moss 2004; Russell and Zomerdijk 2005). Some of these regulatory proteins, and their effects on the Pol I transcription machinery, are listed in Table 10.2.

10.4.2 Regulation of rDNA Chromatin

In addition to these mechanisms that modulate the activity of the Pol I transcription machinery, epigenetic regulation of the rDNA chromatin, which determines the number of active rRNA genes, can also influence the level of rRNA produced. Such epigenetic regulation is thought to be stably propagated throughout cell divisions to ensure that an appropriate proportion of active and inactive rDNA repeats is maintained. The importance of this to nucleolar integrity, genomic stability and the global regulation of gene expression has been proposed (Espada et al. 2007; Guetg et al. 2010; Ide et al. 2010; McStay and Grummt 2008; Paredes and Maggert 2009).

Table 10.2 Positive and negative regulators of cell growth target the Pol I transcription machinery

Regulatory factor	Targets in Pol I transcription machinery	References
Activators		
G1-specific cyclin/CDKs	UBF	Voit et al. (1999) and Voit and Grummt (2001)
ERK	RRN3, UBF	Stefanovsky et al. (2001) and Zhao et al. (2003)
RSK	RRN3	Zhao et al. (2003)
CK2	UBF, RRN3	Lin et al. (2006), Panova et al. (2006), and Bierhoff et al. (2008)
mTOR	RRN3, UBF	Hannan et al. (2003), Claypool et al. (2004), and Mayer et al. (2004)
CBP	UBF	Pelletier et al. (2000)
PCAF	SL1	Muth et al. (2001)
TIP60	UBF	Halkidou et al. (2004)
c-Myc	SL1	Arabi et al. (2005) and Grandori et al. (2005)
RasL11a	UBF	Pistoni et al. (2010)
Repressors		
p53	SL1	Zhai and Comai (2000)
RB/p130	UBF	Cavanaugh et al. (1995), Voit et al. (1997), and Hannan et al. (2000)
CK2	SL1	Panova et al. (2006)
PTEN	SL1	Zhang et al. (2005)
p14ARF	UBF, TTF-I	Ayrault et al. (2006) and Lessard et al. (2010)
GSK3β	SL1	Vincent et al. (2008)
AMPK	RRN3	Hoppe et al. (2009)
JNK2	RRN3	Mayer et al. (2005)

One of the key factors in establishing the epigenetic state of rRNA genes in mammals is TTF-I, which, in addition to its role as a transcription terminator and potential regulator of rDNA topology, can define active or inactive rDNA conformations through its association with chromatin remodelling complexes (reviewed by McStay and Grummt 2008). Epigenetic silencing by TTF-I is mediated by its recruitment of the nucleolar remodelling complex (NoRC) to the promoter-proximal T_0 element. The NoRC subunit TIP5 interacts with TTF-I, and NoRC in turn recruits DNA methyltransferases DNMT1 and DNMT3, and the histone deacetylase-containing Sin3 complex, which mediate transcriptional repression (Santoro and Grummt 2005; Santoro et al. 2002; Zhou et al. 2002). Methylation of a single CpG dinucleotide at the mouse rDNA promoter seems particularly important for transcriptional silencing, as this diminishes binding of the activator UBF to the rDNA (Santoro and Grummt 2001, 2005). NoRC function is dependent on the association of TIP5 with pRNAs. These 150–300 nucleotide transcripts are derived

from the IGS by Pol I-driven transcription from the spacer promoters of a subset of hypomethylated rRNA genes (Mayer et al. 2006, 2008 ; Santoro et al. 2010). Such transcripts are essential for epigenetic silencing of rDNA. Furthermore, a recent study has shown that pRNA can induce *de novo* methylation of rDNA and transcriptional silencing independently of TTF-I and NoRC, by interacting directly with the T_0 element forming a DNA-RNA triplex which is recognised by DNMT3b (Schmitz et al. 2010). In fact, binding of TTF-I and pRNA to T_0 are mutually exclusive (Schmitz et al. 2010). The levels of pRNA and its association with T_0 vary during S phase progression, suggesting a potential link between pRNA and the transmission of epigenetic rDNA silencing between cell divisions (Santoro et al. 2010; Schmitz et al. 2010). Studies of the molecular basis of nucleolar dominance in plant hybrids, whereby NORs from one parental species are dominant over the other, also suggest an involvement of non-coding RNAs derived from the rDNA IGS in determining a repressive pattern of DNA methylation and histone deacetylation (reviewed by Tucker et al. 2010). Therefore, the involvement of such RNAs in selecting the proportion of transcriptionally silenced rDNA repeats, through epigenetic mechanisms, appears to be evolutionarily conserved.

Mechanisms also exist to maintain a proportion of rDNA repeats in an active chromatin conformation. For instance, binding of TTF-I to the T_0 elements of certain rDNA repeats in mouse cells induces chromatin remodelling and transcriptional activation (Langst et al. 1997). This is mediated by an interaction between TTF-I and the chromatin remodeler CSB (Yuan et al. 2007). Transcriptional activation by CSB is dependent on its intrinsic ATPase activity, and also its association with the histone methyltransferase G9a (Yuan et al. 2007). Therefore, TTF-I is integral in determining whether rDNA repeats adopt an active or an inactive epigenetic conformation. However, it is unclear how TTF-I interacts differentially with these positive and negative regulators of transcription to achieve a precise balance between these alternative chromatin states.

Other factors proposed to maintain rDNA in a euchromatic state include the methyl-CpG binding domain protein MBD3, TAF12-recruited GADD45a and the putative chromatin remodeler CHD7 (chromodomain helicase DNA-binding protein 7). These proteins prevent repressive methylation of the rDNA and/or promote the active demethylation of this region (Brown and Szyf 2007; Schmitz et al. 2009; Zentner et al. 2010). In addition to DNA methylation, the methylation state of the rDNA-associated histones also correlates with transcriptional activity: di- and tri-methylation of Lys4 and mono- and di-methylation of Lys36 of histone H3 mark active repeats, whereas di-methylation of Lys9 of histone H3 is associated with silenced rDNA chromatin. The JmjC domain-containing lysine demethylases JHDM1B, KDM2A and PHF8 associate with rDNA and influence this histone methylation pattern (Feng et al. 2010; Frescas et al. 2007; Tanaka et al. 2010).

UBF is also involved in determining the number of active rDNA repeats (Sanij et al. 2008). However, this does not appear to involve epigenetic modifications of the chromatin, but instead occurs through the ability of UBF to displace histone H1, thus preventing H1-induced chromatin condensation (Sanij et al. 2008). In yeast,

the UBF-related protein Hmo1 localises to active rDNA repeats, and a recent study has demonstrated the importance of this to the maintenance of a transcriptionally-competent chromatin state, established following DNA replication through the Pol I transcription-dependent (and potentially histone chaperone-dependent) displacement of nucleosomes (Wittner et al. 2011).

Although mechanisms directly influencing the activity of the Pol I transcription machinery are important for the modulation of rRNA production in response to growth signals, alterations in the rDNA chromatin are also likely to contribute. For instance, Murayama et al. (2008) have described a complex known as eNoSC (energy-dependent nucleolar silencing complex), which mediates the epigenetic repression of rRNA genes in response to energy deprivation. Furthermore, the lysine demethylase KDM2A targets mono- and di-methylated Lys36 of histone H3 and in this way represses transcription by Pol I in response to starvation (Tanaka et al. 2010). In addition to these dynamic alterations in rDNA chromatin in response to changing metabolic conditions, reducing the number of active rDNA repeats has been proposed to contribute to the down-regulation of Pol I transcription that accompanies differentiation (Sanij and Hannan 2008). Moreover, the proportion of active rDNA repeats varies depending on the developmental stage and cell type, indicating that lineage-specific regulation of the number of actively transcribed rRNA genes could be important for vertebrate development (Haaf et al. 1991; Schlesinger et al. 2009).

10.4.3 Deregulated Transcription by Pol I and Disease

The regulation of Pol I transcription is clearly a crucial feature of normal cellular growth and proliferation. The importance of such stringent control is highlighted by the fact that transcription by Pol I is deregulated in various disease states. Most notably, pre-rRNA levels are elevated in a wide range of tumour types and this is thought to be a general feature of human cancers (reviewed by Ruggero and Pandolfi 2003; White 2008). Inactivation of tumour suppressors, aberrant activation of oncogenes (many of which target the Pol I transcription machinery directly as outlined in Table 10.2) and loss of rDNA methylation, are all thought to play a role in this abnormal activation of rDNA expression, contributing to the uncontrolled cell growth and division that is characteristic of tumour cells. In addition, elevated transcription by Pol I underlies the hypertrophic growth of cardiomyocytes, which is a characteristic feature of various cardiovascular disorders (Brandenburger et al. 2001). In contrast, decreased rRNA production, as a result of rDNA promoter hypermethylation in the cerebral cortex, has been described as a feature of the neurodegeneration that accompanies Alzheimer's disease (Pietrzak et al. 2011). Furthermore, the demethylase PHF8 might link dynamic histone methylation at rDNA to mental retardation with cleft lip and palate (Feng et al. 2010).

The consequences of deregulated Pol I transcription during development are highlighted by recent findings regarding the genetic basis of Treacher Collins Syndrome, which can be caused by mutations in Pol I/III subunits or the UBF-interacting

protein Treacle (Dauwerse et al. 2011; Valdez et al. 2004). This craniofacial autosomal-dominant disorder is characterised by a deficiency in neural crest cells, resulting from inadequate ribosome production during development. Abnormalities in ribosome biogenesis give rise to a variety of other congenital disorders, emphasizing the importance of understanding the mechanisms impinging upon ribosome production (Narla and Ebert 2010).

10.5 Conclusions

Transcription by Pol I underlies fundamental cellular functions. Our understanding of this process has grown in recent years, revealing unanticipated complexity. An increasing number of functions are being attributed to the Pol I-specific transcription factors, unravelling the means by which they facilitate rRNA synthesis. Furthermore, many additional factors are now known to bind and regulate this machinery. Recent insights into the intricacies of the structural and topological organisation of rDNA further demonstrate the multifaceted cellular control mechanisms which impinge upon rRNA production. Such elaborate regulation highlights the importance for precise control of transcription by Pol I, a point further emphasized by the apparently universal deregulation of rRNA expression in human tumours. Despite the ever-expanding list of factors and epigenetic control mechanisms that influence transcription by Pol I, the full implications of many of these discoveries have yet to be established. Therefore, further research directed towards resolving the many unanswered questions regarding the complex interplay between regulatory mechanisms targeting the rDNA chromatin and the Pol I transcription machinery is essential, not only to enhance our understanding of cellular growth controls, but also to enable the development of prognostic tools and therapeutic strategies for disease.

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Chapter 11
The RNA Polymerase II Transcriptional Machinery and Its Epigenetic Context

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Abstract RNA polymerase II (Pol II) is the main engine that drives transcription of protein-encoding genes in eukaryotes. Despite its intrinsic subunit complexity, Pol II is subject to a host of factors that regulate the multistep transcription process. Indeed, the hallmark of the transcription cycle is the dynamic association of Pol II with initiation, elongation and other factors. In addition, Pol II transcription is regulated by a series of cofactors (coactivators and corepressors). Among these, the Mediator has emerged as one of the key regulatory factors for Pol II. Transcription by Pol II takes place in the context of chromatin, which is subject to numerous epigenetic modifications. This chapter mainly summarizes the various biochemical mechanisms that determine formation and function of a Pol II preinitiation complex (PIC) and those that affect its progress along the gene body (elongation). It further examines the various epigenetic modifications that the Pol II machinery encounters, especially in certain developmental contexts, and highlights newer evidence pointing to a likely close interplay between this machinery and factors responsible for the chromatin modifications.

11.1 Introduction

The general transcription machinery of a eukaryotic cell constitutes the nuts and bolts through which gene regulation is largely achieved. In recent years, epigenetic modifications that are acquired by a given gene have come to be seen as providing

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the major blueprint for how its transcriptional program will be executed over the longer term. However, the actual translation of the program into actionable output remains the task of the general transcriptional machinery.

In addition to RNA polymerases, this machinery consists of a series of initiation factors that both confer specificity and help launch the polymerase so that it can actually transcribe the body of the gene. During this phase the polymerase is associated with a set of elongation factors that, in addition to ensuring processivity, regulate polymerase progress across the gene through various checkpoints and hurdles that include nucleosomes. The overall process of transcription is thus characterized by a multitude of factors that dynamically interact with the polymerases.

Yet the machineries responsible for the apparently distinct processes underlying transcription on the one hand and epigenetic modifications on the other might intersect, both structurally and functionally. This chapter will mainly describe the workings of the general transcription machinery. It will also briefly examine some selected examples that illustrate the potential for interactions of this machinery with epigenetically modified chromatin and factors responsible for these modifications. Finally, it will touch upon some important biological consequences of such interactions.

11.2 Transcription Is a Multistep Process

Given the complexity of their genomes, eukaryotic cells possess three RNA polymerases, each dedicated to the transcription of a specific class of genes (Roeder and Rutter 1969). Two additional variant polymerases have also been identified in plant cells (Pikaard et al. 2008). RNA polymerase II (Pol II), the enzyme responsible for transcription of genes encoding proteins and some small RNAs, will constitute the subject of this chapter. However, it should be kept in mind that the mechanisms by which the various polymerases assemble into initiation–competent complexes at their promoters are highly analogous and are mediated by functionally equivalent, as well as shared, factors. Thus, much of what we currently know about Pol II would potentially be of relevance to the other polymerases, each of which likely operates within its own particular epigenetic environment.

Pol II genes are under the control of numerous transcription factors that carry regulatory information emanating for example, from developmental, hormonal or pharmacological cues (Brivanlou and Darnell 2002). Typically these factors bind within the vicinity of the gene promoter and through direct or indirect interactions with the factors associated with Pol II (see further below) they parlay the signals into appropriate transcriptional responses. Interestingly, and relevant to the present topic, chromatinization of the template dictates that the activation process take place, broadly, in two steps (Roeder 1998, 2005). In the first step (Fig. 11.1), the transcription factors, in concert with chromatin factors (discussed below), act to breach the chromatin barrier. It is only in the second step that Pol II gains access to its cognate promoter. It has been argued that in contrast to the relatively straightforward transcriptional activation pathways in prokaryotes, chromatin has imparted a distinct

logic to transcriptional regulation in eukaryotes (Struhl 1999). The latter have thus both evolved strategies to deal with the chromatin and developed mechanisms that couple chromatin remodeling with the transcription process *per se*. Otherwise, the core transcriptional machineries of prokaryotes and eukaryotes display a remarkable degree of evolutionary conservation (Cramer 2002; Ebright 2000).

11.3 Pol II and the Preinitiation Complex

Pol II is a 12-subunit enzyme whose central mass consists of the two largest subunits (RPB1 and RPB2) that are orthologs of prokaryotic RNA polymerase components β and β′. Together, they generate a jaw-like structure with a cleft running along its length (Cramer et al. 2008). RPB3 and RPB11, the orthologs of the two prokaryotic α subunits, are located distal to the cleft. Overall, the central core of Pol II is virtually indistinguishable from the corresponding prokaryotic structure (Cramer et al. 2000; Zhang et al. 1999). The remaining (relatively small) subunits, which are mostly eukaryote-specific but include RPB6, the ortholog of prokaryotic ω subunit (Minakhin et al. 2001), are dispersed around this core. The active site, which is responsible for templated incorporation of nucleotides into the growing RNA chain, consists of highly conserved residues and catalytic magnesium ions buried deep within the cleft (Cramer 2002).

Although catalytically competent, Pol II, like prokaryotic RNA polymerases that are dependent on cognate sigma factors, is incapable of accurately initiating from promoter-directed transcription start sites (TSS) in the absence of general transcription factors (GTFs) that include TFIIA, TFIIB, TFIID, TFIIE, TFIIF, and TFIIH (Hahn 2004; Orphanides et al. 1996; Roeder 1996; Thomas and Chiang 2006). The primary role of these GTFs is thus to accurately position and orient Pol II on the promoter and to facilitate initial access of its catalytic site to the transcribed strand of the template. Recall that promoters essentially consist of some combination of a series of defined DNA elements in the vicinity of the TSS (Juven-Gershon et al. 2008). These elements include the TATA box (located at –30 relative to the TSS) and the initiator (+1), as well as more recently defined motifs such as MTE, DPE, and DCE that are mostly located downstream of the TSS. Together with Pol II, the GTFs assemble into a preinitiation complex (PIC), a key intermediate in the transcription activation pathway. To a very large extent, it is the formation and function of the PIC that controls gene activity (Roeder 1998, 2005).

A high-resolution structure of a complete Pol II PIC has not yet been described (Kornberg 2007). However, multiple lines of evidence, including gel mobility shift studies (Buratowski et al. 1989; Flores et al. 1992) and chemical cross-linking (Chen and Hahn 2004; Eichner et al. 2010) have provided detailed insights into how it is assembled and organized, at least on TATA-containing core promoters. PIC assembly begins with the binding of the TBP subunit of TFIID to the TATA box via minor groove interactions (Burley and Roeder 1996). On TATA-less promoters, the TAF components of TFIID are thought to contribute via interactions with any of the

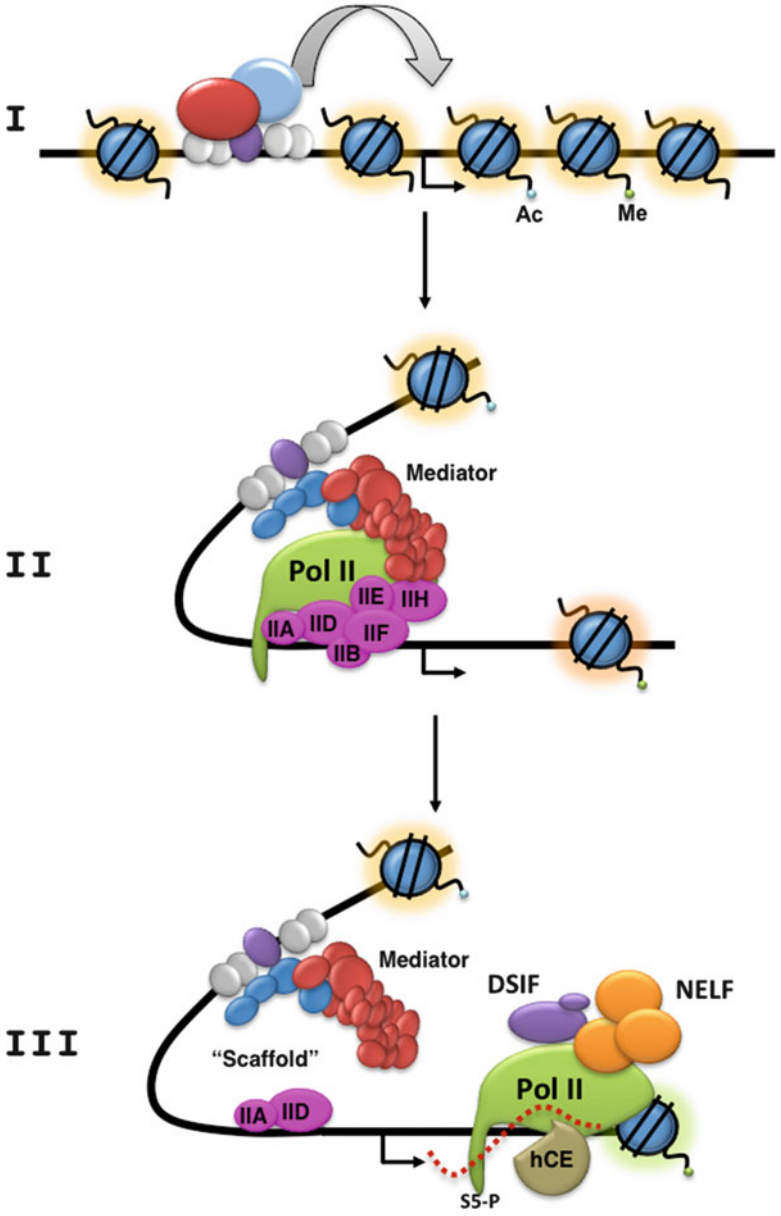

Fig. 11.1 Transcription by Pol II is a multi-step process. The figure depicts a simplified transcription activation pathway that commences when transcriptional activators bind to their cognate sites in the regulatory region of the gene that may be buried in chromatin. These factors recruit a series of chromatin coactivators that can both covalently modify nucleosomes at specific histone residues and mobilize the nucleosomes via ATP-requiring reactions. The resulting intermediate (I) contains chromatin that is characterized by distinct covalent modifications such as acetylation (Ac) and methylation (Me); some nucleosomes may be "evicted". The activators then recruit Mediator (intermediate II). Although the intact Mediator consisting of the core and the kinase module might

other motifs that might be present in the promoter (Burke and Kadonaga 1997; Martinez et al. 1995). In some cases, as-yet-unidentified factors might additionally be required (Martinez et al. 1998). TBP binding to promoter DNA leads to an unusual distortion and sharp bending of the template, which potentially contributes to the final topology of the PIC. TFIIA, which binds upstream of the TATA box, greatly facilitates this interaction. Additional stabilization comes from the interaction of the C-terminal core domains of TFIIB, which makes contacts both with the underside of TBP (Nikolov et al. 1995) and with DNA upstream and downstream of the TATA box, which constitutes the B-recognition element (BRE) (Lagrange et al. 1998). The N-terminal domain of TFIIB makes intimate contacts with Pol II (Kostrewa et al. 2009; Liu et al. 2010), which is recruited to the PIC in conjunction with TFIIF (Flores et al. 1992). In this way, Pol II is positioned accurately and in the correct orientation. TFIIE and TFIIH are the last to enter the PIC and play active roles in the firing of the PIC (Buratowski et al. 1989; Flores et al. 1992; Ohkuma and Roeder 1994).

As to the generality of action of the GTFs, data from genome-wide analyses are not comprehensive enough at this time to conclude whether all the GTFs are required at all genes. Nonetheless, studies of TBP paralogs (TRFs) have revealed that in certain developmental contexts, canonical TFIID might be replaced with a pared down counterpart consisting of TRF3 and at most one TAF (TAF3) (Deato et al. 2008; Deato and Tjian 2007).

11.4 PIC Function

PIC assembly is followed by a series of well-orchestrated events that ultimately lead to RNA synthesis in earnest. The PIC can be thought of as being the counterpart of the prokaryotic “closed complex”. Therefore, promoter melting, the process in which the double-stranded DNA around the TSS is partially unwound, is a prerequisite for the onset of RNA synthesis. Of the two ATPases/helicases present in TFIIH, ERCC3 (XPB) has been implicated in promoter melting (Lin et al. 2005; Tirode et al. 1999). However, unlike conventional helicases, TFIIH seems to unwind promoter DNA through a novel torsional mechanism that entails ATP hydrolysis (Kim et al. 2000). TFIIB and TFIIE also contribute to promoter melting,

Fig. 11.1 (contiuned) be recruited with subsequent loss of the kinase module as the PIC matures, only the core Mediator is shown. PIC assembly entails entry of the various GTFs (TFIIA, TFIIB, TFIID, TFIIE, TFIIF, and TFIIH) and Pol II. After transcription initiation (abortive initiation, which accompanies this step is not illustrated), Pol II clears the promoter. Prior to fully entering the elongation phase (see Fig. 11.2), Pol II, which by now is phosphorylated at Ser5, may pass through a capping checkpoint. The scaffold complex (intermediate III) containing a subset of GTFs and Mediator remains behind at the promoter and can contribute to subsequent rounds of transcription. At the capping checkpoint, Pol II becomes associated with elongation factors including DSIF and NELF. The capping enzyme (hCE) modifies (7MeG) the nascent RNA. Pol II is released from this pause through recruitment of P-TEFb (see Fig. 11.2 legend)

the former most likely through stabilization of the melted state via interactions with the non-template strand (Kostrewa et al. 2009). Melting takes place in multiple steps. Initially, a region extending from −9 to +2 is unwound (Holstege et al. 1997). Subsequently, the melted region ("bubble") is extended, first to +4, and later further downstream, to allow templated phosphodiester bond formation to occur. The discontinuous nature of the bubble extension and early transcription events is particularly noteworthy. Thus, as in prokaryotic systems, the nascent RNA is not extended right away. Rather, abortively synthesized short RNAs are first generated in a stoichiometric excess. It is believed that this is a result of the strains that the PIC is subjected to in this step (Pal et al. 2005). As the strain is periodically relieved through bubble collapse, transcription is aborted leading to release of the short RNAs. At the structural level, this can also be attributed to the N-terminal B-finger/B-reader of TFIIB that reaches into the active site of Pol II, where it can sterically clash with the nascent RNA (Bushnell et al. 2004; Kostrewa et al. 2009; Pal et al. 2005). Because of the divergent demands on the system (bubble collapse versus RNA chain extension), multiple attempts are needed for ensuring that long RNA chain synthesis will eventually win out in so far as TFIIB is involved. It therefore seems also to play an important role in ensuring an orderly transition into the productive phase of transcription by overseeing the process of promoter escape.

Strictly speaking, promoter escape refers to the process whereby Pol II relinquishes its initial contacts within the PIC and begins to acquire a conformation that is more typical of the elongation phase. This process, too, entails multiple substeps. As the RNA is extended past circa +9, the PIC begins to undergo extensive rearrangements (Holstege et al. 1997). In addition to Pol II movements and associated changes in the template structure, the PIC loses a subset of GTFs. While there is some uncertainty as to which subunits are lost, the most convincing data come from studies in yeast and indicate that following Pol II escape a "scaffold" complex consisting of TFIIA, TFIID, TFIIE, TFIIH, and Mediator (below) is left behind; TFIIB is likely shed while TFIIF travels with Pol II (Yudkovsky et al. 2000). The resulting scaffold can serve as an efficient platform for assembly of complete PICs in subsequent rounds of transcription requiring only TFIIB and TFIIF. With regard to the mechanisms underlying promoter escape, in addition to the above-mentioned contribution of TFIIB, the TFIIH-associated ATPase activity likely plays a dominant role in the escape (Lin et al. 2005). However, the precise mechanisms remain uncharacterized.

A hallmark of the escaping Pol II is that it undergoes phosphorylation at the Serine 5 residues of an amino acid sequence ($YS_2PTS_5PS_7$) that is present in highly repeated copies (26 in yeast; 52 in mammals) at the C-terminal end of the RPB1 subunit (CTD) (Buratowski 2009; Egloff and Murphy 2008). Ser5 phosphorylation is carried out by another enzymatic subunit of TFIIH, CDK7. However, it is important to note that, at least in experimental systems reconstituted from purified components, this modification is not likely to be a cause of Pol II escape (Akoulitchev et al. 1995; Gu et al. 1999; Li and Kornberg 1994). Nonetheless, in vivo, the phosphorylation could contribute to this step, for example, by weakening some protein-protein interactions that anchor Pol II in the PIC (Naar et al. 2002). As discussed

below, it is more likely that this modification represents a "mark" of a Pol II molecule that has successfully completed the process of initiation and is now competent to respond to factors involved in transcription elongation and related processes (below). Indeed, highly phosphorylated Pol II is incapable of entering the PIC necessitating dephosphorylation by, among others, Rtr1 (Mosley et al. 2009) and the TFIIF-dependent FCP1 phosphatases (Kobor et al. 1999).

11.5 Factors Affecting Pol II Transcription Post-initiation

After Pol II clears the promoter, it remains subject to numerous additional controls, this time through general transcription elongation factors (Saunders et al. 2006; Sims et al. 2004). The so-called capping checkpoint is the first major regulatory site encountered by Pol II. Most eukaryotic mRNAs bear a 7-methyl guanosine modification at their 5′ ends, which is appended by the capping enzyme at this point as the nascent RNA begins to emerge from the Pol II RNA exit channel. This step coincides with a transient pause that Pol II undergoes following association with the elongation factors DSIF and NELF. Pol II resumes transcription when the negative effects of DSIF and NELF are reversed through the action of a positively acting elongation factor P-TEFb, which may be recruited via an activator (Rahl et al. 2010) and as part of a much larger complex, the "super elongation complex" (SEC), which contains additional elongation factors (Smith et al. 2011). The CDK9 kinase subunit of P-TEFb phosphorylates multiple factors in the paused elongation complex. Its targets include both NELF and DSIF. Phosphorylation of NELF leads to its release whereas phosphorylation of the Spt5 subunit of DSIF transforms it into a positively acting elongation factor that goes on to travel with Pol II further downstream (Fig. 11.2). P-TEFb also phosphorylates the Ser2 residue of the RPB1 CTD, a modification that also is important in coupling transcription elongation to post-transcriptional processes.

Perhaps the most obvious impediment faced by the elongating Pol II is the nucleosome (see further below). Even though the intrinsic forces generated by the advancing Pol II allow it to make some headway into the chromatin (Hodges et al. 2009), the relatively tight wrapping of template DNA around the nucleosome necessitates involvement of additional elongation factors (Sims et al. 2004). These factors use diverse mechanisms that range from mobilization of the nucleosome to alterations of the catalytic properties of Pol II. Among the factors representing the former mechanism are the numerous ATP-dependent chromatin-remodeling factors (such as the SWI/SNF and I-SWI class complexes) that physically displace the nucleosomes (Mohrmann and Verrijzer 2005). Additionally, chaperones, of which FACT is the best characterized, also disrupt the nucleosome by acting as acceptors for displaced histones (Winkler and Luger 2011). Representing the latter mechanism for facilitating transcription through a nucleosome is SII (Guermah et al. 2006; Kireeva et al. 2005), which stimulates the intrinsic RNA cleavage activity of Pol II (Kettenberger et al. 2003). Pol II stalled within nucleosomes tends to backtrack and lose its register; RNA

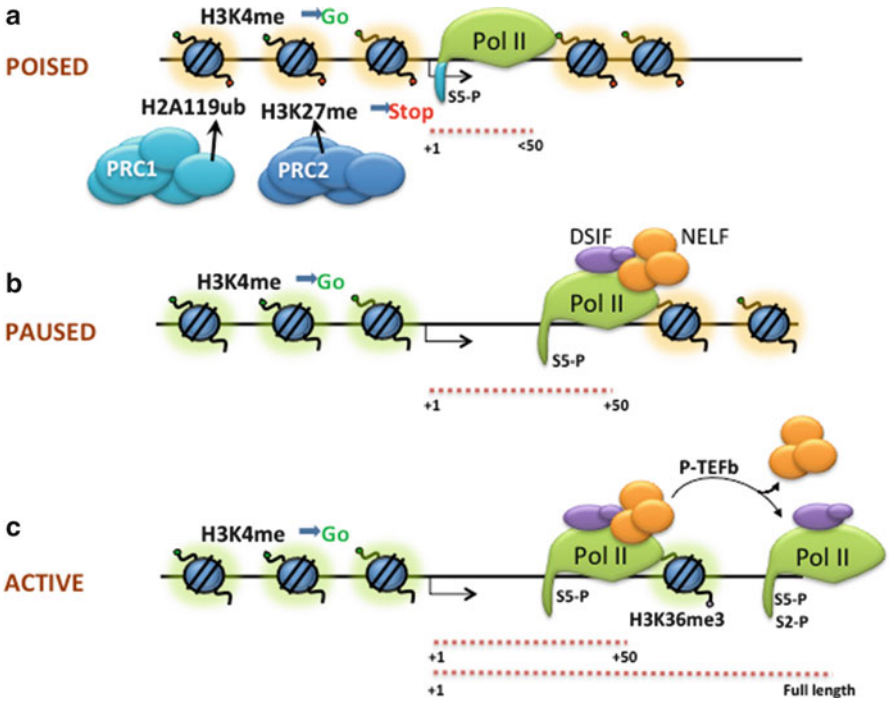

Fig. 11.2 Control of Pol II elongation. (**a**) Genes such as those in ES cells that contain bivalent domains, which are marked by simultaneous methylation of histone H3 at lysine 4 and lysine 27, are characterized by a *poised* Pol II. The poised Pol II is located very close to the TSS (<+50) and is phosphorylated at Ser5 of the CTD, but not Ser2. It is in a conformation that is refractory to detection by antibodies against unmodified CTD. Targets of PRC1 also present ubiquitinated H2A, which through unknown mechanisms has been suggested to restrain Pol II in a poised configuration at these genes. (**b**) Other genes, which might be functionally inactive, carry a *paused* Pol II. While this Pol II is also at a promoter-proximal location, this location is typically somewhat further downstream (circa +50) relative to the poised Pol II in (**a**). The paused Pol II is phosphorylated at Ser5 and may be associated with the elongation factors DSIF and NELF. (**c**) At highly transcribed genes, the pause is released through the phosphorylation of NELF and DSIF by P-TEFb, which leads to NELF release whereas phosphorylated DSIF becomes a positively-acting elongation factor that travels with Pol II. The actively elongating Pol II is further modified at Ser2 and recruits the HMT Set2 that in turn methylates histone H3 at lysine 36 during the elongation phase

cleavage provides the enzyme another chance to go through the blockage. Interestingly, SII appears also to enter the promoter-bound PIC suggesting that it might have additional as-yet-uncharacterized roles in transcription initiation (Kim et al. 2007). At least one other multisubunit elongation factor (PAF1), which has been shown to function in cooperation with SII (Kim et al. 2010), appears to act at the elongation stage both by modulating Pol II elongation rates as well as a device for coupling elongation to the specific epigenetic environment of the gene as discussed below. Although multiple factors that facilitate Pol II transit across this barrier have already been identified, it is likely that given the rate limiting nature of this step – and the potential for regulation – other factors remain to be discovered.

More recently, genome-wide analyses in metazoans have revealed another important post-initiation checkpoint of sorts. These studies show that regardless of whether or not a full-length RNA is transcribed from them, an apparently "paused" Pol II is detectable at a promoter-proximal location (circa +50) at a large number of genes (Margaritis and Holstege 2008; Muse et al. 2007; Zeitlinger et al. 2007). The current interpretation of this observation is that, analogous to what was originally observed for heat-shock genes (Lis 1998), the paused Pol II molecules are likely to reflect species that have initiated but are stalled, potentially in anticipation of a stimulatory signal that would allow them to transition into a full-fledged transcription elongation complex (Nechaev and Adelman 2008). Whereas the precise factorology for this phenomenon has not yet been worked out, nor has it become clear how it relates to the capping checkpoint, there are indications that mechanisms entailing NELF-DSIF-P-TEFb interplay as well as potentially novel pathways might be involved (Muse et al. 2007).

As evident from the preceding discussion, a hallmark of the Pol II transcription cycle is the dynamic association of distinct classes of factors with Pol II. To a significant extent, this is determined by the CTD phosphorylation status of Pol II. It was noted above that Pol II that is competent for PIC formation is essentially in an unphosphorylated state. By the time it arrives at the capping checkpoint it has already acquired the Ser5 modification, which in fact is a prerequisite for interaction with the capping enzyme (Fabrega et al. 2003). Similarly, the actively elongating Pol II is further modified at Ser2. This modification mediates interactions with additional elongation factors (including Spt6 (Sun et al. 2010) and Set2 (Kizer et al. 2005)), as well as RNA processing factors, including those responsible for polyadenylation and termination (Meinhart et al. 2005). In this way, the CTD also is a device for coupling transcription to post-transcriptional processes. Ser7 phosphorylation has also been described and serves to recruit the Integrator complex (a potential transcription cofactor whose precise functions remain uncharacterized) to the atypical U1 and U2 genes (Egloff et al. 2007). In addition to serine phosphorylation, tyrosine phosphorylation (Baskaran et al. 1993), proline isomerization (Xu and Manley 2004) and serine and threonine glycosylation (Kelly et al. 1993) can also occur. Most recently, arginine methylation at one of the non-consensus CTD repeats has also been described (Sims et al. 2011). Together, these modifications have been construed as delineating an underlying "CTD code" that provides readable instructions to the Pol II-interacting factors (Egloff and Murphy 2008).

11.6 Involvement of General Cofactors in Pol II Transcription: Central Role of the Mediator Complex

The general transcription machinery discussed above can function autonomously, especially in what is referred to as "basal" transcription in in vitro studies of transcription (Roeder1996, 1998). However, in the cellular context the machinery is designed to be responsive to regulatory transcription factors. Despite the potential

for direct interactions with components of the general machinery, transcriptional activators typically function through intermediary factors, or coactivators (Roeder 1998). As mentioned, given the chromatin environment, it is not surprising that many coactivators are actually chromatin remodeling factors. Importantly, there is a class of coactivators that by virtue of their close association with the general transcriptional machinery function at the level of PIC. Of these, the 30-subunit Mediator is perhaps the most prominent (Malik and Roeder 2010).

Multiple lines of investigation have converged to establish that Mediator is the major point of control for the Pol II PIC. Originally isolated in yeast (Kornberg 2005; Lee and Young 2000) as a Pol II-interacting cofactor, it has also been shown to interact with numerous transcriptional activators over the years, especially in extensive studies done in metazoan systems (Blazek et al. 2005; Boyer et al. 1999; Fondell et al. 1996; Naar et al. 1999). The earliest models for its function therefore postulated that it interacts as an interface between activators bound to their cognate elements and the PIC assembled at the core promoter (Malik and Roeder 2000). In this capacity its main role has been seen as facilitating recruitment of Pol II to the PIC (Fig. 11.1). However, as argued below, the multifarious properties that have thus far been ascribed to the Mediator favor an alternative view in which it acts as a hub for processing regulatory information. Further, in this view, rather than acting as a binary switch for turning transcription on (or off) it acts to deliver finely calibrated output to the PIC (Malik and Roeder 2010).

Structurally, the Mediator, many of whose individual subunits have been conserved from yeast to human (Bourbon 2008), displays a modular organization. Three main modules (head, middle, tail) constitute the major form of the Mediator. A fourth module, containing the CDK8-cyclin C pair, as well as two other large subunits, reversibly associates with the bulk Mediator. Association of this "kinase" module, confers generally negative properties on the Mediator although recent reports have suggested dual (i.e., both positive and negative) functions for it (Donner et al. 2007; Furumoto et al. 2007).

Broadly, whereas some of the resident subunits in the head and tail have been implicated in Pol II (and associated) interactions (Cai et al. 2009; Soutourina et al. 2011; Takagi et al. 2006), subunits in the tail are typically responsible for interactions with transcriptional activators (Malik and Roeder 2010). Through such interactions, specific Mediator subunits have been implicated in distinct cellular pathways. Most prominently, the MED1 subunit (its precise modular location is not yet mapped, but it is likely to lie close to the tail (Malik and Roeder 2010)) serves as the target for several nuclear receptors (Ito et al. 2000), which because of their responsiveness to hormonal signals are major regulators of animal physiology. In this way, the MED1 subunit channels transcription signals borne by the receptors to the PICs formed on their target genes. Ultimately, this may be translated into the appropriate cellular response. The classic illustration of this high degree of specificity in Mediator action comes from analyses of a model developmental system comprised of mouse embryo fibroblasts (MEFs) isolated from embryos of mice in which the gene encoding MED1 has been ablated (Ge et al. 2002; Ito et al. 2000). Although *MED1*$^{-/-}$ mice are embryonic lethal (reflecting the importance of the subunit in the

overall development of the animal), MEFs isolated from such embryos just prior to death can be maintained in culture (reflecting the subunit's dispensability for housekeeping gene expression). Importantly, while wild-type MEFs can be differentiated into adipocytes through stimulation of PPARγ receptor-dependent pathways, *MED1*$^{-/-}$ MEFs fail to undergo such differentiation. Other Mediator subunits, notably MED15 (Yang et al. 2006), MED23 (Stevens et al. 2002) and MED12, a component of the kinase module (Carrera et al. 2008), also control discrete cellular pathways.

Although the coactivator role of Mediator was initially emphasized, it has become clear that it also affects basal (i.e., activator-independent) transcription (Baek et al. 2006; Mittler et al. 2001; Takagi and Kornberg 2006). Indeed, genome-wide analyses have revealed a near-universal requirement for Mediator at all genes, at least in yeast (Holstege et al. 1998). Interestingly, in this system and in contrast to the conditional requirement for MED1 mentioned above, deletion of a core (head) subunit is as deleterious as deletion of a Pol II subunit such that transcription of a vast majority of genes in the cell is affected. Also of note, multiple physical and functional interactions between Mediator and GTFs have been documented. These include Mediator's ability not only to recruit TFIIH to the PIC (Esnault et al. 2008; Pavri et al. 2005) but also its effects on the CTD phosphorylation function of TFIIH. Mediator effects on TFIIH can be both positive (Kim et al. 1994) and negative, the latter through phosphorylation of cyclin H in TFIIH by the CDK8 kinase (Akoulitchev et al. 2000). Further, efficient TFIIB recruitment to the PIC is also dependent on prior Mediator association (Baek et al. 2006). Mediator and TFIID also physically interact (Johnson et al. 2002). Thus, in the updated view, Mediator may even be regarded as a bona fide GTF.

Even more intriguingly, Mediator's role in PIC function might also potentially extend to post-initiation steps. Genetic data in yeast originally provided evidence for common roles of the MED31 subunit and SII (Guglielmi et al. 2007) as well as for links between this subunit and the Set2 complex, which can also function as an elongation factor (Krogan et al. 2003b). Functional interplay between Mediator and the elongation factor DSIF was also reported (Malik et al. 2007). Similarly, there are indications that Mediator interactions with P-TEFb and other SEC components might affect transcription elongation (Donner et al. 2010; Takahashi et al. 2011). Thus, Mediator might be uniquely situated to tie together the PIC formation, initiation, and elongation phases of transcription. In this light, an outstanding question is whether (and how) Mediator might also regulate the promoter-proximal paused Pol II.

In addition to its effects on the PIC, Mediator also operates within the context of multiple coactivators that impinge on the control regions of the gene. The emerging theme is that Mediator might coordinate the function of these diverse factors (Malik and Roeder 2010). For the most part, these are chromatin coactivators that help to "loosen" up the template prior to PIC assembly. Recall that the nucleosome, the fundamental unit of chromatin, is composed of two copies each of four core histones, H2A, H2B, H3, and H4, around which 146 bp of DNA is wrapped (Luger et al. 1997). The N-terminal tails of histones are relatively accessible to enzymatic modifications such as acetylation, methylation, phosphorylation, ubiquitination and

sumoylation (Kouzarides 2007). Of all known histone modifications, acetylation is the only modification that directly causes the structural relaxation of chromatin by introducing a negative charge, favoring the recruitment of transcription factors as well as the process of transcription. Other histone modifications mediate the recruitment of activating and repressing effector complexes that ultimately mediate: (a) acetylation or deacetylation of histones; (b) nucleosome mobility, or even nucleosome eviction, to facilitate the compaction or relaxation of chromatin typically via mechanisms entailing ATP hydrolysis; and (c) recruitment of structural non-histone proteins that participate in chromatin compaction and assembly of higher order structures that are typical of heterochromatin.

Among the best-characterized chromatin cofactors that participate in short-term activation of genes are the various ATP-dependent remodelers like the p300 and GCN5 acetyltransferases. GCN5 is part of a larger complex (SAGA) that also includes certain TAFs and might function as a core promoter factor in lieu of TFIID at some promoters (Rodriguez-Navarro 2009). Additional factors (mostly methyl transferases and demethylases) whose actions lead to changes of a more long-term nature ("epigenetic") are discussed further below. The mechanisms of action of p300 and GCN5 nicely exemplify how histone modification is closely tied to Mediator and indeed Mediator might be involved in facilitating transitions between the chromatin machinery and the PIC (Black et al. 2006; Liu et al. 2008; Wallberg et al. 2003). Thus, although our simplified models for transcription activation describe it as a two-step process, Mediator likely provides continuity between them.

11.7 Negative Control of the Pol II Machinery

Although activation mechanisms have been emphasized here, it must be remembered that negative control is an integral component of the logic of gene regulation in eukaryotes. Indeed, "anti-repression" can add an additional layer of control to the overall activation of any given gene (Roeder 1998). Most obvious is the negative control exerted at the level of the chromatin structure, both through the nucleosomes themselves and through additional interacting proteins (e.g., linker histones) that further compact and condense the chromatin. Additional factors that contribute at this level include chromatin-modifying enzymes, especially histone deacetylases, or HDACs, and other co-repressors, which may be recruited in a gene-specific manner to distinct loci (Smith and Workman 2009). Here we point out some of the more salient mechanisms that most directly impact on the Pol II machinery and include a series of negative cofactors that target distinct GTFs. The dual (positive and negative) features of the Mediator have already been alluded to above. Other factors include NC2 and Mot1, which impact on TFIID. NC2 can interact with TBP and sterically occlude TFIIA and TFIIB from entering the PIC (Kamada et al. 2001). Mot1, on the other hand, is an ATPase that actively displaces TBP from promoters (Auble et al. 1994). Surprisingly, at some loci, these factors manifest as positive factors. In the case of NC2, it stimulates transcription from DPE-containing

promoters at the expense of TATA-containing promoters (Willy et al. 2000). It has been suggested that NC2 affects the conformation of the TBP-TATA complex and thus mobilizes TBP towards weaker DPE-containing promoters (Schluesche et al. 2007). Similarly, Mot1 might also act to displace TBP from cryptic promoters and make it available to weak, but bona fide, promoters (Sprouse et al. 2008).

11.8 RNA Pol II Function Is Linked to Histone Modifications

As is evident from the above discussion of how Pol II and its associated factors function, the presence of densely packed nucleosomes offers a physical barrier for the efficient recruitment and processivity of Pol II. This supports the notion that genes immersed in highly packed areas of chromatin are less likely to engage RNA polymerase into productive rounds of transcription. Yet, more recent discoveries intriguingly suggest that histone modifications, especially those associated with epigenetic effects, can also play a direct role in modulating Pol II recruitment and processivity.

Most convincingly, genome-wide occupancy studies have revealed striking correlations between the presence of Pol II and certain histone modifications. Thus, transcriptional initiation strongly correlates with histone H3 trimethylated at lysine 4 (H3K4me3) and acetylation of histone H3 at lysine 9 and 14 (H3K9,14 ac) at nucleosomes near the TSS (Bernstein et al. 2002; Guenther et al. 2007; Santos-Rosa et al. 2002; Schubeler et al. 2004). Transcription elongation correlates with the presence of H3 trimethylated at lysine 36 (H3K36me3) at nucleosomes located in the body of the gene (Bannister et al. 2005; Kharchenko et al. 2010). Whether the presence of these marks is a consequence of transcription, or whether they play an active role in Pol II recruitment and processivity is presently unclear. Nonetheless, the Set1 complex, which catalyzes the H3K4me3 modification in yeast, is recruited to actively transcribed genes by interacting with the RNA Pol II-associated PAF1 complex (Krogan et al. 2003a). Experiments in yeast further suggest that H3K4me3 modification is deposited after Pol II recruitment and phosphorylation of Ser5 at the CTD. Accordingly, the recruitment to chromatin of the MLL1 complex, a mammalian homolog of the yeast Set1 complex, might in fact occur through the direct interaction of MLL components with the CTD of PoI II that has been phosphorylated at Ser5 (Hughes et al. 2004). This not only suggests that the establishment of the H3K4me3 mark is a consequence of PoI II recruitment but that Pol II is an active player in the process. On the other hand, specific TAFs in TFIID can bind directly to the H3K4me3 mark and this interaction seems potentiated by H3K9 and K14 acetylation (Vermeulen et al. 2007). This therefore suggests that H3K4me3 could also actively participate in PIC formation, and hence Pol II recruitment.

The multifunctional Mediator complex has also been implicated in establishing the epigenetically silenced status of certain neuronal genes in extraneuronal cells. In this system, the MED12 subunit of the generally repressive Mediator kinase module contributes to long-term repression of the target gene through interactions with multiple factors that include the G9a methyl transferase, which methylates H3K9 (Ding et al.

2008). This modification (H3K9me2) is known to attract heterochromatin protein 1 (HP1), which in turn is recognized by the DNA-methylating enzyme DNMT1. Together with other repression mechanisms that also operate at these loci (Ooi and Wood 2007), repression of the target genes is thus ensured through subsequent generations. Conversely, and reflecting the dual modes of function of this module, the MED12 and MED13 subunits of the Mediator interact with the Pygopus factor in Drosophila, which is a PHD finger-domain-containing protein that recognizes H3K4me3, the mark associated with transcriptional activation (Carrera et al. 2008; Fiedler et al. 2008).

As for histone modifications near the TSS, transcriptional elongation by Pol II also may influence epigenetic modifications in the body of the gene. For example, the deposition of H3K36me3 along the gene body in actively transcribed genes is a consequence of the recruitment of the histone methyltransferase (HMT) Set2 by the elongating Pol II. It is believed that methylated H3K36 acts in part to prevent cryptic initiation within genes through the recruitment of histone-deacetylase activities (Lee and Shilatifard 2007).

11.9 Epigenetic and Transcriptional Regulation in ES Cells: A Case Study

Transcription regulation in embryonic stem (ES) cells, with their unusual potential for both self renewal and pluripotency, illustrates how an intricate choreography entailing the Pol II machinery, on the one hand, and the epigenetic machinery, on the other, can lead to dramatic biological outcomes.

Chromatin immunoprecipitation (ChIP) coupled to DNA microarray analyses have revealed that in human ES cells, the phenomenon of promoter-proximal pausing of Pol II is widespread. Thus, 75% of all annotated promoters are enriched for Pol II and the H3K4me3, H3K9,14 ac epigenetic marks. However, only about half of these produce detectable transcripts and show elongation-associated H3K36me3 (Guenther et al. 2007), suggesting that successful elongation is a rate-limiting step for productive transcription. Further, a recent study using the new technique of GRO-seq (Min et al. 2011), in which nascent transcripts are mapped, confirms that promoter-proximal pausing of Pol II is a potential rate-limiting step for at least 40% of the genes in ES cells (as well as in MEFs, another cell type that was analyzed). Even at highly transcribed genes, Pol II occupancy is markedly higher near the TSS compared to the coding region. However, relative Pol II occupancy at TSS versus the coding region decreases with increasing gene activity, suggesting that overcoming the paused configuration of Pol II contributes towards overall activation of the gene. Comparison of GRO-seq signals between ES and MEFs also revealed that both Pol II recruitment (PIC formation) and transcriptional elongation contribute to the differential gene expression seen in these cell types (Min et al. 2011).

In ES cells, a prominent group of genes that recruit Pol II, but are not actively transcribed, are those encoding developmental regulators (Min et al. 2011; Stock et al. 2007). These genes display peculiar modification patterns, called bivalent domains,

which are characterized by the presence of large stretches of chromatin containing H3K27me3 that in turn harbour smaller regions of H3K4me3 around the TSS (Fig. 11.2). The coexistence of these two antagonistic marks, one (H3K27me3) associated with transcriptional repression and the other (H3K4me3) with activation, has been suggested to play a role in silencing developmental genes in ES cells while keeping them poised for activation upon initiation of specific developmental pathways (Azuara et al. 2006; Bernstein et al. 2006). The K27me3 modification is catalyzed by the Polycomb group of proteins (PcG), which, in mammals, are found in at least two distinct complexes. The Polycomb repressive complex 2 (PRC2) contains four core components including the SET domain-containing EZH2 or EZH1 subunits, which are able to trimethylate H3K27. This mark is specifically recognized by the chromodomain of CBX proteins present in the Polycomb repressive complex 1 (PRC1), which also contains, among other subunits, a RING domain protein that mediates the ubiquitylation of histone H2A (Sauvageau and Sauvageau 2010).

Bivalent domains can be classified into two types based on whether they contain both PRC1 and PRC2, or only PRC2 (Ku et al. 2008). Genes occupied by PRC1 in ES cells usually encode for transcription factors and morphogenesis molecules that play key roles in development. GRO-seq data confirms that genes with bivalent domains are transcribed at very low levels. However, and especially in the case of genes bound by PRC2 only, notable levels of transcriptionally engaged Pol II are observed near their TSS, compared to the greatly reduced levels of productively elongating Pol II (Min et al. 2011). Genome-wide studies in Drosophila melanogaster also indicate that many genes that contain Polycomb responsive elements (PRE) proximal to the TSS produce short transcripts suggesting that transcription starts but elongation fails (Kharchenko et al. 2010). Interestingly, genes encoding regulatory and developmental functions are overrepresented in this class and they are further marked with H3K27me3 and either H3K4me1 or H3K4me2 but not H3K4me3. However, GRO-seq data in ES cells suggest that Pol II at bivalent gene promoters is confined to extremely 5′ proximal regions (Min et al. 2011). Whereas this again suggests that Pol II is stalled at these promoters, it appears that it is in a conformation that is different from that of the paused Pol II observed on actively transcribed genes (Stock et al. 2007). It can therefore be referred to as being in a "poised" conformation although precise structural features remain unidentified (Fig. 11.2). This poised Pol II is phosphorylated at Ser5 of the CTD but not at Ser2. Indeed, it cannot be detected with standard antibodies against CTD, suggesting that its conformation is different from that of the paused Pol II that is more widely observed. Further, ablation of Ring1B, the PRC1 subunit that mediates ubiquitination of histone H2A, results in loss of ubiquitinated H2A at bivalent genes and de-repression of these genes without changes in H3K27me3. Interestingly, the levels of Ser5 and Ser2 phosphorylation of Pol II at these genes does not change, but Pol II is now detectible with antibodies against CTD, suggesting that a conformational change has released Pol II from the poised state (Stock et al. 2007). Therefore, Ring 1-mediated ubiquitination of H2A seems to restrain poised Pol II at bivalent genes. The precise role of the H3K4me3 mark at bivalent genes, especially in relation to Pol II recruitment, remains unclear. Knock-down of selected H3K4 demethylases in

ES cells causes an increase in the H3K4me3 levels and the de-repression of certain bivalent genes (Adamo et al. 2011; Pasini et al. 2008). In contrast, ES cells null for Jarid2, a subunit of PRC1 involved in its recruitment to target genes, fail to recruit Pol II to bivalent genes while levels of H3K4me3 are unchanged (Landeira et al. 2010).

In summary, several histone modifications seem to be directly correlated with the presence of Pol II at the TSS or coding regions of genes notably, although not exclusively, in developmental systems. While the details of the interplay between these modifications and Pol II function are still not clear, the recent findings discussed above show that histone modifications play direct roles in regulating the transcriptional machinery.

11.10 Conclusions

The past two decades have witnessed a major expansion in our knowledge of how the Pol II machinery functions. Numerous factors that allow it to assemble into an active PIC at gene promoters as well as factors that act as it is navigating the gene body have been identified and characterized. In many cases, structural and biochemical studies have combined to reveal, at an unprecedented level of detail, how these factors impact Pol II function. With more recent studies linking Pol II function with specific epigenetic marks in the genome, the latest challenge in the field now is to describe in equivalent detail how the Pol II machinery plays out in this context. Epigenetic modifications have also been described in terms of a "histone code", which is laid down by so-called "writers" and subsequently interpreted by "readers" (Jenuwein and Allis 2001; Strahl and Allis 2000). As discussed here, the Pol II machinery seems to contain both writers and readers. Future work is sure to reveal further how other individual components of this elaborate machinery are affected by and, in turn, influence the epigenetic landscape, and ultimately lead to interesting biological outcomes.

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Chapter 12
RNA Polymerase III Transcription – Regulated by Chromatin Structure and Regulator of Nuclear Chromatin Organization

Chiara Pascali and Martin Teichmann

Abstract RNA polymerase III (Pol III) transcription is regulated by modifications of the chromatin. DNA methylation and post-translational modifications of histones, such as acetylation, phosphorylation and methylation have been linked to Pol III transcriptional activity. In addition to being regulated by modifications of DNA and histones, Pol III genes and its transcription factors have been implicated in the organization of nuclear chromatin in several organisms. In yeast, the ability of the Pol III transcription system to contribute to nuclear organization seems to be dependent on direct interactions of Pol III genes and/or its transcription factors TFIIIC and TFIIIB with the structural maintenance of chromatin (SMC) protein-containing complexes cohesin and condensin. In human cells, Pol III genes and transcription factors have also been shown to colocalize with cohesin and the transcription regulator and genome organizer CCCTC-binding factor (CTCF). Furthermore, chromosomal sites have been identified in yeast and humans that are bound by partial Pol III machineries (extra TFIIIC sites – ETC; chromosome organizing clamps – COC). These ETCs/COC as well as Pol III genes possess the ability to act as boundary elements that restrict spreading of heterochromatin.

12.1 Introduction

Transcription in eukaryotes is carried out by multiple DNA-dependent RNA polymerases with specialized functions (Pol I, Pol II and Pol III in animal cells and in addition Pol IV and Pol V in plant cells). Being composed of 17 subunits, RNA

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polymerase III (Pol III) displays the most complex protein composition of these RNA polymerases. Pol III transcribes genes encoding small untranslated RNAs that are characterized by a limited number of promoters (three major types in mammals) and are accordingly recognized by general transcription factors (reviewed in Geiduschek and Kassavetis 2001; Huang and Maraia 2001; Schramm and Hernandez 2002; Roeder 2003; Dumay-Odelot et al. 2010). Many of these genes are conserved from unicellular to multicellular organisms (5S RNA; tRNAs; U6 RNA; RNAse P and RNAse MRP RNAs; 7SL RNA). These RNAs participate in the regulation of different aspects of gene expression, including transcription, RNA processing, translation and protein translocation to the endoplasmic reticulum. Other genes are transcribed by Pol III in a variety of eukaryotes from protozoa to metazoa, including the genes coding for vault RNAs, Y RNAs and the 7SK RNA (Dieci et al. 2007). Higher eukaryotes contain in addition a variable number of Pol III-transcribed retrotransposed elements (small interspersed nuclear elements -SINEs) that constitute a substantial fraction of the DNA of a given genome (about 11% of the human genome; Cordaux and Batzer 2009). Besides these Pol III-transcribed genes encoded by eukaryotic genomes, several virus-encoded Pol III transcription units have been described that require the transcription apparatus of the host to be expressed (adenovirus-associated VA genes and Epstein-Barr virus-associated EBER genes). In yeast, Pol III promoters are essentially located downstream of the transcription start site (TSS), although sequences 5′ the TSS have been described that influence transcription efficiency and even start site selection *in vitro* and *in vivo* (Eschenlauer et al. 1993; Giuliodori et al. 2003). In mammals, type 1 (5S gene) and type 2 promoters are internal to the gene (e.g. tRNA; VA genes), whereas type 3 promoters (e.g. U6; 7SK; RNAse P; RNAse MRP genes) are entirely located upstream the TSS (Dieci et al. 2007; Orioli et al. 2012). Transcription factors recognizing these genes and being required for their expression have extensively been reviewed (Dumay-Odelot et al. 2010; Geiduschek and Kassavetis 2001; Huang and Maraia 2001; Schramm and Hernandez 2002) and they are schematically represented in Figs. 12.1 and 12.2 for human type 2 and type 3 promoters.

For a long time, Pol III transcription has been known to be essential for the survival of cells, but data supporting the participation of Pol III transcription in the regulation of cell fates or disease have only emerged recently (reviewed in Dumay-Odelot et al. 2010; Marshall and White 2008; White 2008). Some of these functions appear to be intimately connected to the regulation of chromatin dynamics and nuclear architecture. Here, we will describe our current knowledge of the genes and proteins that are involved in these processes.

In general, two functional connections of Pol III transcription with chromatin configuration and nuclear architecture can be discerned. First, the regulatory function of chromatin structure on Pol III transcription activity. Second, the contribution of Pol III genes or Pol III promoter elements to the organization of the genome within the nucleus.

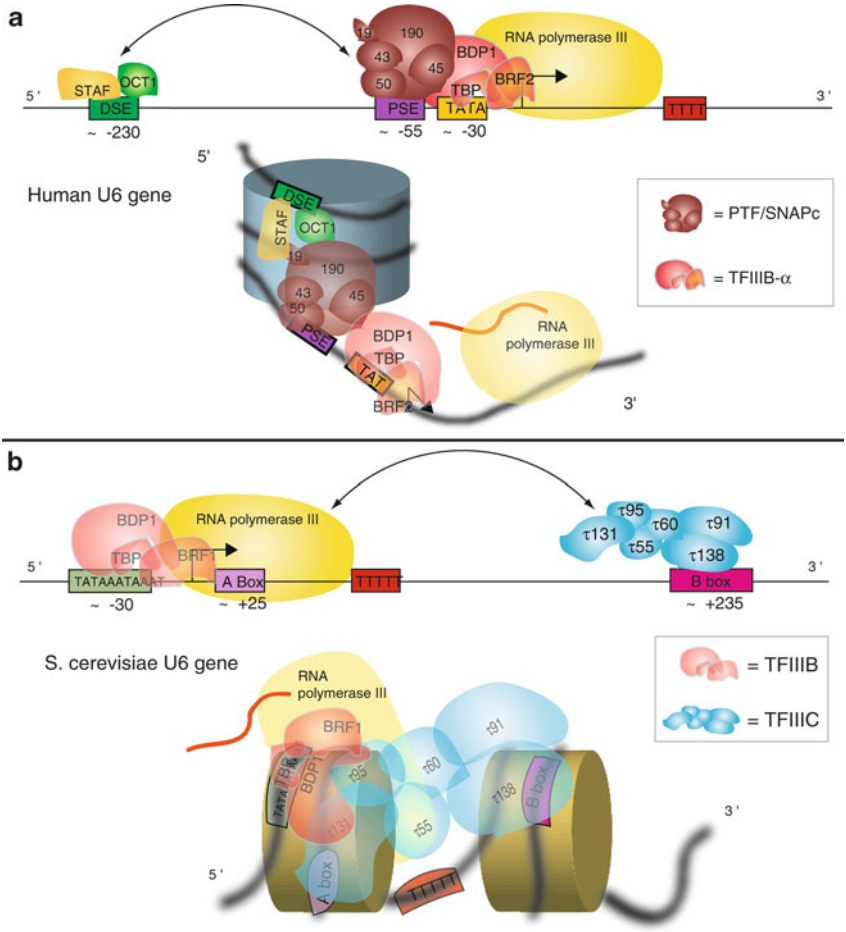

Fig. 12.1 Transcription of the *S. cerevisiae* and of the human U6 gene is facilitated by nucleosomal organization of their promoter/enhancer elements. (**a**) The human U6 gene regulatory sequences are composed of the distal and proximal sequence elements (DSE and PSE), as well as a TATA-box. Factors required for the transcription of the U6 gene are STAF, OCT1, PTF/SNAPc (subunits of 190, 50, 45, 43 and 19 kDa) and TFIIIB-α (subunits BDP1, TBP and BRF2; reviewed in 1). The *upper panel* shows a symbolized representation of U6 gene regulatory elements and transcription factors on linear DNA, indicating that contacts of DSE-bound STAF/OCT1 transcription factors with the PSE/TATA-bound basal Pol III transcription machinery (PTF/TFIIIB-α, RNA polymerase III) cannot be established without physically approaching these elements/factors. The *lower panel* shows a model of a nucleosome positioned in between DSE and PSE (Stünkel et al. 1997; Zhao et al. 2001), permitting the establishment of direct contacts of OCT1/STAF with the basal Pol III machinery and transcription initiation. Nascent RNA is symbolized by a *red line*. (**b**) The *S. cerevisiae* U6 gene regulatory elements are composed of a TATA-like box 5′ of the transcription initiation site, a gene-internal *A Box* and a *B Box* downstream the transcription termination site (Burnol et al. 1993a, b; Eschenlauer et al. 1993; reviewed in Teichmann et al. 2010). RNA polymerase III is recruited to the *S. cerevisiae* U6 gene by TFIIIC (composed by the tau subunits 138, 131, 95, 91, 60 and 55) and TFIIIB (composed by TBP, BDP1 and BRF1). The *upper panel* indicates that TFIIIC-TFIIIB interactions on a linear U6 template are limited due to the distance in between A and B boxes (symbolized by a *line* with two *arrowheads*). The *lower panel* shows a model of a positioned nucleosome that approaches A- and B-Boxes, thereby facilitating contacts of TFIIIC with both elements leading to the recruitment of TFIIIB and productive transcription (Burnol et al. 1993a, b). Nascent RNA is symbolized by a *red line*

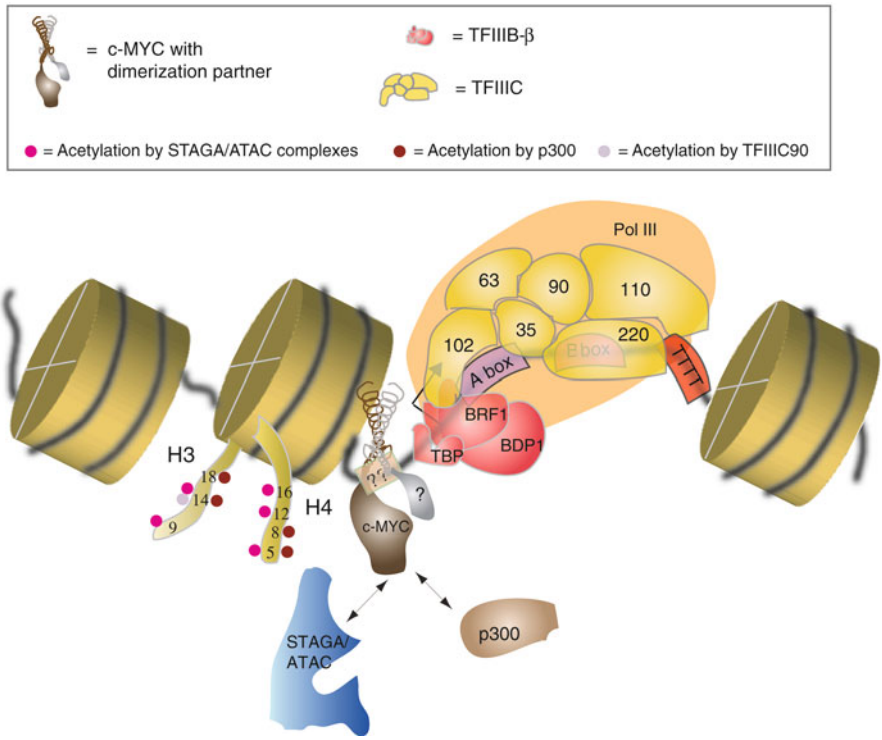

Fig. 12.2 Model of the activation of type 2 promoter (tRNA) transcription by c-MYC in the context of nucleosomal organization of promoter elements. Recognition of an unidentified DNA sequence close to Pol III genes (E box?) by c-MYC, probably heterodimerized with a yet unknown transcription factor (MAX?) leads to the recruitment of the histone acetyltransferases (HATs) GCN5 (in SAGA or ATAC complexes) and/or p300 (Gomez-Roman et al. 2003; Kenneth et al. 2007; Mertens and Roeder 2008; reviewed in Dumay-Odelot et al. 2010). These HATs acetylate the appropriately indicated lysine residues in histone tails (for simplicity, only one tail is shown for histones H3 and H4, respectively). The basal Pol III transcription machinery, composed of TFIIIC (subunits 220, 110, 102, 90, 63 and 35), TFIIIB-β (components BDP1, TBP and BRF1) and Pol III itself. TFIIIC90 contributes to acetylation of histone H3K14 (Hsieh et al. 1999)

12.2 Regulation of Pol III Transcription in Chromatin

Transcription by RNA polymerase III has best been studied in the yeast *S. cerevisiae* and in human cells. All proteins that are essential for the transcription of Pol III genes *in vitro* in these organisms have been identified and the corresponding cDNAs have been cloned. Thus, *in vitro* transcription systems on 'naked' DNA have been described that are reconstituted from highly purified recombinant or epitope-tagged transcription factors and RNA polymerase III itself. In *S. cerevisiae*, it has been shown that tRNA genes can be transcribed by recombinant TFIIIC (six subunits), recombinant TFIIIB (three subunits) and recombinant Sub1 (orthologous to mammalian PC4),

together with affinity-purified Pol III (Ducrot et al. 2006; Tavenet et al. 2009). A similar *in vitro* system has been described for transcription of the adenoviral VA1 gene with human factors. In this system, recombinant TFIIIC has been replaced by affinity-purified TFIIIC (Dumay-Odelot et al. 2007; Mertens and Roeder 2008; Haurie et al. 2010; transcription factors required for the expression of type 2 promoters are schematically shown in Fig. 12.2; PC4 is omitted from this Figure since its mode of action is not known). These yeast and human *in vitro* transcription systems suggest that the essential basal Pol III transcription factors have been identified.

In the past few years and based on the knowledge of the basal Pol III machinery, research of gene expression by Pol III has developed into new directions. First, the influence of the chromatin and of modulators thereof on the expression of Pol III genes has been analyzed. Second, genome-wide analyses have been undertaken for determining the presence of basal factors and of chromatin regulators at Pol III genes in living cells.

12.2.1 The Impact of Chromatin on Pol III Transcription

Pioneering experiments demonstrating the influence of chromatin on the regulation of Pol III transcription were carried out shortly after the discovery of three nuclear DNA-dependent RNA polymerases (Roeder and Rutter 1969). It was shown that isolated nuclei (Weinmann and Roeder 1974) or chromatin prepared from mouse myeloma cells (Marzluff and Huang 1975) retained the ability of faithfully transcribing 5S and tRNA genes. Chromatin-derived transcription of the 5S gene could be specifically enhanced by exogenously added Pol III, indicating that the chromatin contained factors that were required for transcription of the gene and that allowed the recruitment of Pol III (Parker and Roeder 1977). Assembly of nucleosomes with purified histones and DNA by employing a salt dialysis method showed that these basal units of chromatin inhibited transcription (by Pol I and Pol II), even in the absence of linker histones (Wasylyk et al. 1979). Preassembly of the Pol III preinitiation complex in such a chromatin assembly system, however, prevented repressive effects of chromatin (Gottesfeld and Bloomer 1982; Felts et al. 1990; Tremethick et al. 1990). Minichromosomes isolated by sucrose gradient centrifugation confirmed the repressive effect of chromatin on 5S RNA transcription, but also showed that a similarly prepared minichromosome carrying the $tRNA_i^{Met}$ gene remained accessible for transcription (Lassar et al. 1985).

The importance of promoter recognition by Pol III transcription factors with respect to transcription in the context of chromatin *in vivo* was first demonstrated for the *S. cerevisiae* SNR6 (U6 RNA) gene. TFIIIC-binding to a B Box element downstream the transcription termination site was essential for transcription of the U6 gene in reconstituted chromatin *in vitro* as well as *in vivo* (Burnol et al. 1993a, b; Fig. 12.1a). Although the promoter context of the U6 gene in humans is distinct from that found in *S. cerevisiae* (Teichmann et al. 2010) and transcription of this gene does not require TFIIIC (Waldschmidt et al. 1991), it was also shown to depend

on a specific nuclosomal configuration. A nucleosome specifically positioned in between distal and proximal sequence elements was demonstrated to be a central determinant for the expression of not only the U6 (Stünkel et al. 1997; Zhao et al. 2001; Fig. 12.1b), but also the 7SK gene (Boyd et al. 2000).

In contrast, the gene-internal tRNA promoters were shown to be nucleosome-free or able to prevent nucleosome assembly if being actively transcribed. By employing nucleosome positioning signals, artificially directing a nucleosome over the A Box of a tRNA gene *in vivo* it was demonstrated that ongoing Pol III transcription prohibited nucleosome delivery over the tRNA promoter (Morse et al. 1992). Later on, by genome-wide localization, it was shown that most tRNA genes reside in nucleosome-free regions in *S. cerevisiae* (Albert et al. 2007) and *P. falciparum* (Westenberger et al. 2009). The histone variant H2A.Z was shown to be present at the 5′ end of genes transcribed by Pol II or Pol III and to regulate the formation of a nucleosome-free region over the transcription start site (Zhang et al. 2005; Albert et al. 2007; Marques et al. 2010; Mahapatra et al. 2011). In the case of the tRNA SUP4 gene, the histone chaperone FACT, together with the HMG-box protein NHP6 and chromatin remodeling complex RSC were shown to be involved in depositing and removing H2A.Z from gene-flanking nucleosomes (Mahapatra et al. 2011). It was furthermore demonstrated that chromatin structure affects transcription of the ZOD1 gene without altering the occupancy of this gene by Pol III transcription factors (Guffanti et al. 2006). Taking into account that NHP6 is involved in establishing nucleosomal configurations that are permissive for Pol III transcription (Mahapatra et al. 2011) and that NHP6 requirement for transcription increases if Pol III gene-flanking regions comprise suboptimal TFIIIB binding sites (Braglia et al. 2007), it may well be that nucleosomal reorganization at the ZOD1 gene is dependent on NHP6. Such a possible action of NHP6 at the ZOD1 promoter may remove a nucleosome positioned over the 5′ region of the gene, resulting in productive transcription. The importance of nucleosome positioning at tRNA genes was put into a distinct perspective when it was uncovered that tRNA genes do not only represent sites for transcription, but also serve as boundary elements, at least in the yeasts *S. cerevisiae* and *S. pombe*, separating euchromatin from heterochromatin (see also Sect. 12.5).

12.2.2 Regulatory Proteins That Influence Pol III Activity in Chromatin

12.2.2.1 MYC

Robert J. White and colleagues described that the protein encoded by the proto-oncogene *c-MYC* is able of stimulating mammalian Pol III transcription. The activation of Pol III transcription was attributed to direct interactions of c-MYC with the basal transcription factor TFIIIB (Gomez-Roman et al. 2003). However, c-MYC plays several roles in the activation of transcription by RNA polymerase II, including the recruitment of chromatin modifying complexes, most notably those containing the

histone acetyltransferase GCN5 (KAT2A; Flinn et al. 2002). GCN5, together with its essential cofactor TRRAP has been shown to activate Pol III transcription (Kenneth et al. 2007; Fig. 12.2) in a process that is regulated by the ribosomal protein L11 (Dai et al. 2010). Furthermore, c-MYC-dependent depositioning of histone H2A.Z was demonstrated to influence Pol II transcription (Martinato et al. 2008) and a similar function of c-MYC in Pol III transcription cannot be excluded. Thus, c-MYC may contribute to the regulation of Pol III transcription in multiple ways, by affecting the chromatin structure of Pol III-transcribed genes and by the recruitment of the basal machinery to Pol III genes. These actions may critically contribute to c-MYC's ability of participating in cellular transformation (Eilers and Eisenman 2008). Indeed, it was shown that ongoing Pol III activity is required for c-MYC's contribution to transforming primary cells into tumor cells (Johnson et al. 2008). Thus, c-MYC-dependent modulation of chromatin modification and conformation at Pol III gene loci may turn out critical for cellular transformation.

12.2.2.2 Histone (Lysine) Acetyl Transferases (KATs)

Besides the lysine acetyl transferase GCN5 (KAT2A) mentioned above, also p300 (KAT3B) was shown to enhance Pol III transcription (Mertens and Roeder 2008). The ability of KAT3B to stimulate Pol III transcription on *in vitro* assembled chromatin templates was dependent on KAT3B acetyltransferase activity. In addition, KAT3B activated Pol III transcription of a naked $tDNA_i^{Met}$ template as well as TFIIIC-promoter-binding in the absence of Acetyl-CoA. Thus KAT3B may regulate Pol III transcription during the process of chromatin opening and subsequently during preinitiation complex formation and transcription initiation. In addition to KAT2A and KAT3B that both have been best described for their functions in Pol II transcription (Sterner and Berger 2000; Nagy and Tora 2007), a Pol III-specific KAT activity intrinsic to human transcription factor TFIIIC was identified. The TFIIIC90 subunit was shown to specifically acetylate lysine 14 of histone H3, whereas the entire, affinity-purified TFIIIC complex preferentially exhibited H4 acetyltransferase activity (Kundu et al. 1999; Hsieh et al. 1999; Fig. 12.2). However, the H4 acetyltransferase activity could not yet be attributed to a specific TFIIIC subunit and may either be due to altered specificity of TFIIIC in the nucleosomal context or may have been conferred by a protein co-purifying with TFIIIC. Recently, it was furthermore shown that histone H3S28 phosphorylation enhances Pol III transcription in chromatin (Zhang et al. 2011).

12.3 New Ideas from ChIP-Seq

ChIP sequencing (ChIP-seq) performed in several distinct human cell lines provided important information about the occupancy of Pol III loci by RNA polymerase III, suggestive of their expression and also of cell type-specific differences

in their occupation (Barski et al. 2010; Canella et al. 2010; Moqtaderi et al. 2010; Oler et al. 2010; Raha et al. 2010). ChIP-seq data suggest that nucleosomes are depleted over tRNA genes (Moqtaderi et al. 2010), similar to the observations made in yeast (Lee et al. 2007; Mavrich et al. 2008). Histone modifications typical for Pol II transcription start sites (TSS) or Pol II enhancers (H3K4me1; H3K4me2; H3K4me3; H3K4ac; H3K9ac; H3K27ac; H3K36ac; H4K5ac; H4K8ac; H4K91ac; H2B5ac; H2B12ac; H2B120ac; Wang et al. 2008; Zhou et al. 2011) are enriched in the vicinity of Pol III expressed genes (Barski et al. 2010; Oler et al. 2010). Thus, enzymes adding or removing these marks (see Table 12.1) may contribute to the regulation of Pol III transcription. In addition, the histone variants H2A.Z and H3.3 that are associated with actively transcribed Pol II gene regulatory regions (Jin et al. 2009) are also enriched at Pol III genes (Barski et al. 2010; Oler et al. 2010). In agreement with an earlier study (Listerman et al. 2007), Pol II itself also specifically crosslinks close to Pol III TSS. Furthermore, several Pol II transcription activators (STAT1; ETS1; c-JUN; JUN-D; c-FOS; c-MYC) and co-activators (CBP) were crosslinked nearby Pol III TSS (Oler et al. 2010; Raha et al. 2010). In contrast, Pol III genes that were not occupied by its transcription machinery were enriched in histone H3K27me3 (Barski et al. 2010; Oler et al. 2010), a histone mark typically associated with inactive genes (Zhou et al. 2011).

The number of TFIIIC binding sites largely exceeds that of the sites occupied by Pol III (Moqtaderi and Struhl 2004; Moqtaderi et al. 2010). Two thirds of these extra TFIIIC (ETC) sites are found close to Pol II genes (<1 kb). The ETC loci are also associated with histone modifications that are typically found at active Pol II genes. In contrast to transcribed Pol III genes, however, the highest density of these histone modifications is found directly next to the sites of TFIIIC-binding and diminishes with the distance from these sites. This observation indicates that these histone modifications may be introduced in a TFIIIC-dependent manner. Furthermore, TFIIIC co-localizes with CTCF at ETCs and to a lesser extent at transcribed Pol III genes (Moqtaderi et al. 2010; Oler et al. 2010), indicating a role for TFIIIC in the organization of the chromosomes within the nucleus (Fig. 12.3; see also Sect. 12.4.2).

Taken together, the ChIP-seq studies from a variety of human cell lines demonstrated not only that Pol III enrichment at its target promoters, but also that TFIIIC-binding to ETCs coincides with nucleosomal environments that are favourable for transcription, including typical histone modifications and the presence of specific histone variants.

12.4 Nuclear Organization by Pol III

Increasing evidence suggests that genes transcribed by Pol III are clustered within the nucleus and may be involved in the spatial organization of the nucleus. Most data on Pol III gene clustering have been obtained in the yeasts *S. cerevisiae* and

Table 12.1 Histone modifications being associated with (**A**) or excluded from (**B**) genes in contact with the Pol III transcription machinery (Barski et al. 2010). Enzymaes that have been shown to be involved in the respective modifications have been indicated to the right. With the exception of GCN5 (KAT2A; Kenneth et al. 2007) and p300 (KAT3B; Mertens and Roeder 2008), the involvement of these enzymes in the regulation of Pol III transcription has not been documented, but they may represent possible regulators of this transcription system

Modification	Addition by:	Removal by:
(A)		
H3K4me1	NSD3	LSD1; KDM1A
	SET1A, B; KMT2F, G	AOF1; KDM1B
	MLL1-5; KMT2A-E	JARID1B; KDM5B
	Nimura et al. (2010) and Black and Whestine (2011)	Black and Whestine (2011)
H3K4me2	NSD3	LSD1; KDM1A
	SET1A, B; KMT2F, G	AOF1; KDM1B
	MLL1-5; KMT2A-E	JARID1A-D; KDM5A-D
	SET7; KMT7	Black and Whestine (2011)
	SMYD3	
	Nimura et al. (2010) and Black and Whestine (2011)	
H3K4me3	SET1A, B; KMT2F, G	JHDM1B; KDM2B
	MLL1-5; KMT2A-E	JARID1A-D; KDM5A-D
	SMYD3	Black and Whestine (2011)
	PRDM7	
	Nimura et al. (2010), Black and Whestine (2011), and Scharf and Imhof (2011)	
H3K4ac	Mst1; KAT5	HDAC3
	Xhemalce and Kouzarides (2010)	Eot-Houllier et al. (2008)
H3K9ac	GCN5A; KAT2A	SIRT6
	PCAF; KAT2B	Michishita et al. (2008)
	Nagy et al. (2010)	
H3K27ac	CBP; KAT3A	NURD (Reynolds et al. 2011)
	Tie et al. (2009)	
H3K36ac	GCN5; KAT2A	?
	Morris et al. (2007)	
H4K5ac	GCN5A; KAT2A	HDAC3 (Hartman et al. 2005; Demmerle et al. 2012)
	PCAF; KAT2B	
	CBP; KAT3A	
	p300; KAT3B	
	Anamika et al. (2010), Altaf et al. (2010)	
H4K8ac	GCN5A; KAT2A	?
	PCAF; KAT2B	
	MOF; KAT8	
	CBP; KAT3A	
	p300; KAT3B	
	Anamika et al. (2010), Altaf et al. (2010)	

(continued)

Table 12.1 (continued)

Modification	Addition by:	Removal by:
H4K91ac	HAT4 (Yang et al. (2011)	?
H2BK5ac	CBP; KAT3A p300; KAT3B Anamika et al. (2010)	?
H2BK12ac	CBP; KAT3A p300; KAT3B Anamika et al. (2010)	?
H2BK120ac	CBP; KAT3A p300; KAT3B Gatta et al. (2010)	?
(B)		
H3K9me3	Ash1; KMT2H Suv39H1,2; KMT1A,B RIZ1; KMT8 SETDB1; KMT1E Nimura et al. (2010), Black and Whestine (2011), and Scharf and Imhof (2011)	JMJD2; KDM4 Nimura et al. (2010), Black and Whestine (2011), and Scharf and Imhof (2011)
H3K27me3	EZH2; KMT6 NSD3 Nimura et al. (2010), Black and Whestine (2011), and Scharf and Imhof (2011)	JMJD3; KDM6A UTX; KDM6B Nimura et al. (2010), Black and Whestine (2011), and Scharf and Imhof (2011)

S. pombe, but recent data also support Pol III gene-mediated genome organization in human cells. A large proportion of the 274 tRNA genes in the yeast *S. cerevisiae* have been localized to the nucleolus, regardless of their primary genomic localization (Thompson et al. 2003; Wang et al. 2005). This spatial relocation brings them into vicinity of 5S RNA genes that are embedded in the 100–200 rDNA repeats and thus found in the nucleolus. In the yeast *S. pombe*, tRNA genes have been found close to the nuclear periphery, in regions that also contain centromeric DNA. Some of the *S. pombe* tRNA genes are close to centromeres on linear maps of the genome (52 of the 174 fission yeast tRNA genes; Takahashi et al. 1991), but others that are dispersed throughout the three chromosomes have also been found associated with centromeres (Iwasaki et al. 2010). In both cases, *S. cerevisiae* and *S. pombe*, through direct genomic association or through intranuclear relocalization to specific environments, tRNA genes are found in the vicinity of 5S RNA genes. Thus, the nuclear organization of Pol III-transcribed genes may facilitate coordinated expression of tRNA and 5S RNA genes and it may be important for the co-regulation of Pol I and Pol III transcription (Briand et al. 2001).

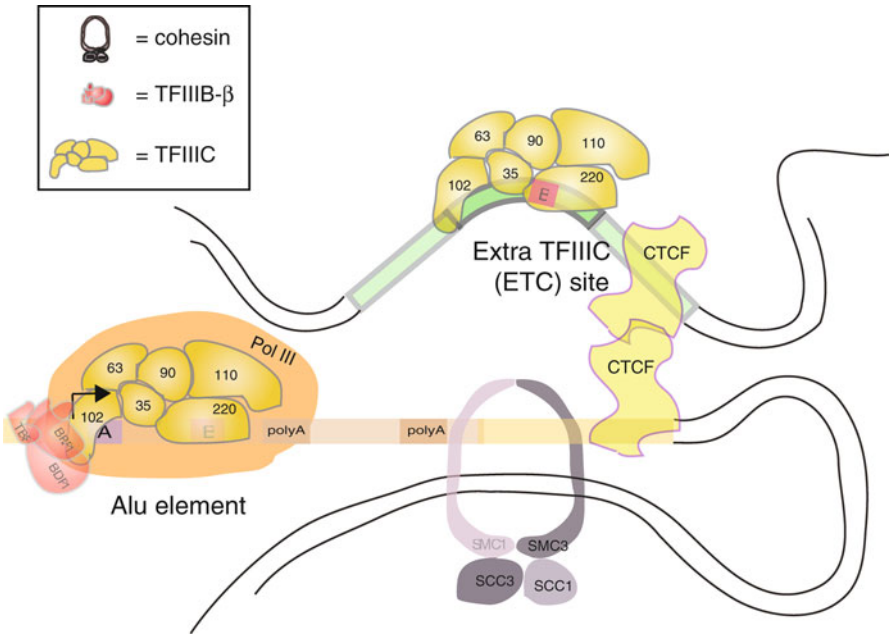

Fig. 12.3 Hypothetical model of a possible contribution of Pol III genes and promoter elements in the establishment of intra- and interchromosomal interactions in mammalian cells. An Alu element is associated with cohesin (Hakimi et al. 2002), which may enable the formation of intrachromosomal loops (Wood et al. 2010). Cohesin associates with CTCF (Parelho et al. 2008; Wendt et al. 2008) and CTCF may form homodimeris or multimers (Phillips and Corces 2009) with CTCF bound to other chromosomal sites (e.g. Extra TFIIIC (ETC) locus (Moqtaderi et al. 2010)). Such multimerizations of CTCF bound to distinct sites may permit the formation of interchromosomal contacts and help to structure nuclear chromatin, possibly in a cell type-specific manner. Subunits of TFIIIC and TFIIIB-β (see Fig. 12.2), as well as of cohesin (SMC1, 3 and SCC 1,3) are appropriately indicated. The ETC is composed of a B box or an unrelated DNA sequence and is bound by TFIIIC, whereas little or no TFIIIB or Pol III are present at this site (Moqtaderi et al. 2010)

12.4.1 Condensin

Subnuclear localization of tRNA genes in the yeast *S. cerevisiae* and *S. pombe* involves the condensin complex (D'Ambrosio et al. 2008; Haeusler et al. 2008; Iwasaki et al. 2010). The condensin complex in budding yeast is composed of two "structural maintenance of chromosomes – SMC" subunits (SMC2 and SMC4) and three non-SMC subunits (Ycs4, Ycs5/Ycg1 and Brn1; Hirano 2005). Vertebrates contain two paralogous condensin complexes composed of five subunits each (the ATPases SMC2 and 4 and auxillary subunits CAPG/G2, CAP-D2/D3 and CAP-H/H2; Hirano 2005; Hudson et al. 2009). In yeast, condensin binding sites are found

close to TFIIIC-binding sites and to the "sister chromatid cohesion" 2 and 4 subunits (SCC2/4) of the cohesin loading complex. SCC2/4 and TFIIIC are thought to assist condensin association with chromosomes (D'Ambrosio et al. 2008). Mutation of condensin subunits resulted in the loss of tRNA clustering at the nucleolus. Direct interactions of TFIIIC, TFIIIB and condensin were suggested by co-immunoprecipitation (Haeusler et al. 2008). The co-localization of condensin with TFIIIC was not restricted to Pol III-transcribed genes, but also occurred at extra TFIIIC (ETC) B Box sequences and even at genomic sites that did not contain clear B Box motifs. In addition, a mutation in the TFIIIC subunit Tfc3 (τ138) resulted in reduced DNA-recruitment of the condensin subunit BRN1 and of its loader SCC4 (D'Ambrosio et al. 2008). Complementary data from the yeast *S. pombe* showed that a mutation in SMC4 (*cut3-477*) led to reduced association of tRNA genes with centromeres (which are often located close to the nucleolus) and at the same time to impaired chromosome condensation. The condensation defect and the tRNA relocation could be rescued by simultaneous introduction of a mutant TFIIIC subunit (orthologous to τ138; *sfc3-1*) that negatively impacts on Pol III transcription (Iwasaki et al. 2010). Thus, in *S. pombe*, reduced Pol III transcription favours the centromeric location of Pol III genes and their association with condensin. Together the data from *S. cerevisiae* and *S. pombe* demonstrate that TFIIIC assists in proper loading of condensin to chromosomes. This function of TFIIIC is also reflected by the presence of condensin at all 274 nuclear *S. cerevisiae* tRNA genes (D'Ambrosio et al. 2008). TFIIIC and possibly TFIIIB, by interacting with condensin and with Pol III genes may play a role in structuring chromatin within the nucleus in yeasts. The clustered nucleolar (*S. cerevisiae*) or centromeric (*S. pombe*) organization of tRNA genes probably involves functional or physical contacts of TFIIIC, SCC2/4 and condensin present at two or more distinct Pol III genes. These contacts may possibly also be established in between tRNA genes and ETC sequences of the same or different chromosomes. According to such a model, the nucleolar localization of tRNA genes in *S. cerevisiae* could be dependent on condensin- interactions with TFIIIC/TFIIIB-complexes that are present on tRNA genes and on condensin being bound in the vicinity of 35S rRNA genes (Lavoie et al. 2004; Wang et al. 2005). Such a hypothetical condensin-dependent interaction of tRNA genes and 35S rRNA genes may, at least in parts, explain why the tRNA genes are often found within the nucleolus of *S. cerevisiae*. Likewise, interactions of centromer-bound and tRNA-bound condensin may help tethering these tRNA genes to centromeres in *S. pombe*.

A similar coupling of condensin and TFIIIC functions in higher eukaryotes has not yet been described. However, several data are not in favour with an absolute evolutionary conservation of these concerted functions of condensin and TFIIIC. For example, yeast do not contain subunits orthologous to vertebrate condensin II, suggesting that condesin II may not be involved in Pol III gene-dependent clustering. In addition, vertebrate condensin I, which is evolutionarily related to yeast condensin is excluded from the nucleus during interphase (Hudson et al. 2009), making it unlikely that it could be involved in similar processes of nuclear organization as those having been discovered in yeast, which comprise condensin functions in

tethering tRNA genes to centromeres during interphase (Iwasaki et al. 2010). Accordingly, clustering of tRNA genes has not yet been reported in vertebrates (Pombo et al. 1999). Although an involvement of condensin in establishing Pol III gene-dependent subnuclear structures in higher eukaryotes cannot firmly be excluded, the above cited data point to alternative mechanisms of nuclear organization in higher eukaryotes, probably relying on genome organizing molecules such as CTCF (see Sect. 12.4.2).

12.4.2 Cohesin

The ring shaped tetrameric cohesin complex is composed of SMC1/3 and SCC 1/3 subunits (Bose and Gerton 2010). As for condensin (D'Ambrosio et al. 2008), loading of cohesin onto chromosomal DNA requires the SCC2/4 loading complex (Ciosk et al. 2000). Accessory proteins including Pds5, Rad61 and Eco1 are involved in cohesin function (Bose and Gerton 2010). In the yeasts *S. cerevisiae* and *S. pombe*, cohesin is often found close to convergently transcribed genes, suggesting that it is translocated to these sites after having been loaded onto chromatin by the SCC2/4 loading complex (Lengronne et al. 2004). Cohesin has functionally been connected to Pol III transcription (Donze et al. 1999; Dubey and Gartenberg 2007). Mutation of *SMC1* or *SMC3* genes led to the loss of tDNA boundary activity at the transcriptionally repressed HMR mating type locus (Donze et al. 1999) (see also Sect. 12.5). Boundary element activity was shown to depend on an actively transcribed $tRNA^{Thr}$ gene, thus linking cohesin function to Pol III transcription (Donze and Kamakaka 2001). Deletion of silent information regulator 3 (SIR3) led to loss of SCC1 association with the HMR locus (Chang et al. 2005). SIR2-4 proteins form a complex that, together with SIR1, is required for the establishment of heterochromatin at the silent mating-type loci. SIR2 is a NAD-dependent histone H4K16ac deacetylase and SIR3 binds to deacetylated H4K16. SIR 3 and SIR 4 are required for heterochromatin spreading (Johnson et al. 2009). Furthermore, deletion of the tDNA, mutation of its B Box or mutation of the BRF1 subunit of TFIIIB reduced cohesion of the HMR locus (Dubey and Gartenberg 2007). Thus, the binding of cohesin to HMR is dependent on a component of the SIR heterochromatin establishment complex and on the recruitment of TFIIIB to the $tRNA^{Thr}$ gene. Moreover, mutation of the chromatin remodelling complex subunit RSC2 also led to reduced cohesion at HMR. In this context, it should be mentioned that a genome-wide analysis demonstrated that RSC is recruited to numerous tRNA genes in *S. cerevisiae* (Ng et al. 2002) and interacts with RNA polymerase III (Soutourina et al. 2006). Interestingly, mutation of the ISW2 subunit of the ISWI complex that is required for tDNA-dependent barrier function at the HMR (Gelbart et al. 2005; Oki and Kamakaka 2005; Tackett et al. 2005) did not result in alteration of cohesion at this locus. Thus, tDNA barrier activity requiring ISWI complex function and cohesin loading depend on distinct chromatin modifying complexes. In summary, the following model may be proposed: Rsc, recruited by tRNA-bound TFIIIB/Pol III is

involved in assisting cohesin loading, which is required for ISWI-dependent tDNA boundary function.

Although tRNAs in mammals have not been shown to co-localize at specific sites within the nucleus and neither condensin nor cohesin association with these genes has hitherto been described, related, but distinct connections of cohesin and Pol III-transcribed genes have been reported. It was shown that human Rad21 (orthologous to the *S. cerevisiae* cohesin subunit SCC1) directly interacts with the Snf2h subunit of the ISWI complex at Pol III-transcribed Alu elements *in vivo* (Hakimi et al. 2002). Furthermore, cohesin was found to co-localize with the CCCTC-binding factor (CTCF) which may be involved in positioning cohesin complexes after having been loaded onto DNA (Nasmyth and Haering 2009). CTCF has been described as a regulator involved in insulation and also in transcriptional activation or repression. It has been shown that CTCF contributes to intra- and interchromosomal interactions, thereby helping to establish higher order chromatin structures (Ling et al. 2006; Kurukuti et al. 2006; Phillips and Corces 2009). CTCF has also been localized close to ETC sites in human K562 cells (Moqtaderi et al. 2010). Thus, cohesin and CTCF have both been linked to human Pol III promoter sequences and they have been shown to colocalize at insulator regions (Parelho et al. 2008; Wendt et al. 2008). These data suggest that some of the underlying mechanisms of establishing subnuclear architectures involving cohesin and RNA polymerase III transcription components have been evolutionary conserved, even if the participation of tRNA genes in this process may have become less evident (a hypothetical model is shown in Fig. 12.3). It should be taken into consideration that yeast and human genomes differ in size (about 3×10^9 base pairs in humans (International Human Genome Sequencing Consortium 2004) versus 1.2×10^7 base pairs in *S. cerevisiae* (Goffeau et al. 1996)). This 250-fold difference in size faces a comparatively minor difference in the number of tRNA genes being in contact with the Pol III transcription machinery (274 in *S. cerevisiae* (Harismendy et al. 2003; Roberts et al. 2003; Moqtaderi and Struhl 2004)) versus about 300 in humans (Barski et al. 2010; Canella et al. 2010; Moqtaderi et al. 2010; Oler et al. 2010; Raha et al. 2010). As a consequence, tRNA genes genes are much more 'diluted' within the human genome (on average about 1 transcribed tRNA gene per 10^7 nucleotides) than in *S. cerevisiae* (one tRNA gene per 5×10^4 base pairs). In view of this difference, it is difficult to imagine that human tRNA genes are implicated in the same condensin- and cohesin-dependent functional structuring of the genome as has been shown in yeast. If ever it may turn out that Pol III genes participate in the organization of the genome in higher eukaryotes including humans, it may well be that repetitive elements such as SINEs (see also below) contribute to this function. The finding that cohesin colocalizes with Alu sequences (Hakimi et al. 2002) supports speculations about such a model.

Mutations in cohesin have been shown to be the underlying cause for genetic diseases. The Cornelia de Lange syndrome (CdLS) and the Roberts Syndrome (RS) are attributed to mutations in proteins affecting cohesin function. Mutations in *SMC1*, *SMC3* and *SCC2* have been linked to CdLS and mutations in *ESCO2* (orthologue of *S. cerevisiae ECO1*; acetyltransferase of SMC3) to RS. CdLS and RS are characterized by mental and growth retardation, including craniofacial

anomalies and the RS in particular by symetric hypomelia (Liu and Krantz 2008). In order to understand the molecular mechanisms leading to CdLS and RD, disease-related mutations in *SCC2* and *ESCO2* have been introduced into the orthologous *S. cerevisiae* proteins (scc2-D730V and eco1-W216G). These mutations lead to chromosome decondensation and changes in nuclear architecture. Importantly, colocalization of tRNA genes and the Pol II-transcribed GAL2 gene was impaired, whereas no apparent phenotype on cohesin binding to and distribution on chromatin, as well as chromatid cohesion could be observed (Gard et al. 2009). The loss of GAL2-tRNA colocalization upon mutation of *SCC2* or *ECO1* may be explained by an impaired ability of mutated cohesin to establish intrachromosomal loops that otherwise may approach the GAL2 gene and tRNA genes and subsequently lead to their nucleolar location (similar to the model of an intrachromosomal loop established by cohesin shown in Fig. 12.3). These data indicate that the development of cohesinopathies may depend on defects in gene expression, possibly involving alterations in Pol III gene-dependent establishment of nuclear architecture.

12.5 tDNA Boundary Function

tRNA genes were shown to act as insulator elements. The term 'insulator element' is employed with regard to describing two distinct mechanisms: First, the insulator element may separate enhancer (or repressor) elements from a basal promoter, thereby hindering enhancer-bound activators (or repressors) from activating (repressing) transcription. A second function of an insulator element is its ability to form a boundary in between hetero- and euchromatin. This mode of action leads to the separation of repressive chromatin structures from those that are permissive for transcription. Both mechanisms have been suggested to operate in the case of tRNA genes.

Concerning the former mechanism, tRNA genes have been shown to influence the expression of close-by located Pol II-transcribed genes. Several lines of evidence have been reported in support of such a Pol III gene-mediated influence on the expression of Pol II-transcribed genes. It has been shown that active transcription of tRNA genes represses Pol II transcription (Kinsey and Sandmeyer 1991; Hull et al. 1994; Kendall et al. 2000). The tRNA mediated gene silencing (tgm) was dependent on nucleolar localization of tRNA genes, since mutants that disrupted nucleolar integrity lead to scattered nuclear tRNA gene distribution, accompanied by the loss of tgm (Wang et al. 2005; Haeusler et al. 2008). Thus, nuclear organization and Pol III transcriptional activity were both demonstrated crucial for the inhibition of Pol II transcription by tRNA genes.

With respect to the second mechanism, it was shown that a $tRNA^{Thr}$ gene restricts the spread of heterochromatin at the HMR mating type locus in *S. cerevisiae* (Donze et al. 1999; Donze and Kamakaka 2001). Similarly, STE6 α2 operator-mediated repression of CBT1 expression was blocked by a $tRNA^{Thr}$ gene in Matα cells

(Simms et al. 2004). In the case of the HMR-E silencer, it was shown that tRNA-mediated silencing depends on several features of the Pol III-transcribed gene. First, the $tRNA^{Thr}$ gene could not be replaced by certain other Pol III-transcribed genes, such as U6 or 5S RNA genes. Second, the spacing of A- and B-Boxes turned out crucial, since an intron-containing tRNA gene could only replace the $tRNA^{Thr}$ gene after deletion of its intron. Third, sequences surrounding the tRNA gene were likewise important for barrier function. Fourth, mutations affecting Pol III transcription abolished heterochromatin barrier. In addition to DNA sequence requirements, mutations in tfc3 (τ138; largest subunit of TFIIIC) or in BRF1 (TFIIIB subunit) impaired barrier activity, whereas a transcription initiation incompetent mutation of Pol III (*rpc31-236*) did not affect insulator function. Interestingly, mutation of GCN5 or SAS2 histone acetyltransferases (HAT) (Donze and Kamakaka 2001) or combined deletion of the $tRNA^{Thr}$ gene and mutation of the EAF3 HAT (Oki and Kamakaka 2005) demonstrated that these HATs cooperate with the $tRNA^{Thr}$ gene to establish the heterochromatin barrier. Together, these data point to a complex barrier mechanism that depends on the tRNA promoter and surrounding sequences, on transcription factor binding and HAT activity. However, mechanisms for establishing barrier activity may vary in between different tRNA genes. In the case of the CBT1 gene, it was shown that histone acetylation at this locus did not change upon deletion of the tRNA gene barrier. Either the tRNA gene barrier at the STE6 α2 operator functions different from that at the HMR locus or the contribution of GCN5 and SAS2 HATs to barrier function of the latter is indirect, possibly involving chromatin remodelling and nucleosome positioning. Indeed, it was demonstrated that an actively transcribed tRNA gene affects nucleosome positioning *in vivo* (Morse et al. 1992). Furthermore, components of Pol III pre-initiation complexes have been shown to interact and to recruit subunits of chromatin remodelling complexes to Pol III genes. The TFIIIB component BDP1 recruits ISW2 to about 50% of the *S. cerevisiae* tRNA genes (Bachman et al. 2005; Gelbart et al. 2005). ISW2 is involved in nucleosomal spacing and in transcription repression (Mellor and Morillon 2004). However, it seems as if ISW2 recruitment does not affect Pol III transcription, but rather impacts on Ty integration upstream the tRNA genes (Bachman et al. 2005). Rsc has also been shown to be recruited to Pol III promoters, independently of their transcriptional activity or the presence of TFIIIB (Ng et al. 2002; Soutourina et al. 2006). Importantly, Rsc together with the HAT Rtt109 (being structurally related to p300; Tang et al. 2008) were shown to be required for nuleosome eviction at the HMR silent mating type locus, creating an about 700 nucleotide nucleosome-free region surrounding the $tRNA^{Thr}$ gene (Dhillon et al. 2009). Full barrier activity at HMR was furthermore dependent on the HMG-box containing protein NHP6 (Braglia et al. 2007). Taken together, the documented recruitment of HAT and chromatin remodelling activities to tRNA genes and their requirement for the establishment of barrier function suggest that basal Pol III gene-bound transcription factors, by recruiting chromatin modifying activities, participate in the remodelling of chromatin leading to the reconfiguration of local chromosome structures.

Not only subnuclear repositioning of tRNA genes, but also barrier functions of tRNA genes have been conserved from *S. cerevisiae* to *S. pombe*. According to its

genomic localization close to the centromere of chromosome 1, cen1, a $tRNA^{Ala}$ gene has been shown to block the propagation of pericentromeric heterochromatin into the flanking euchromatin (Scott et al. 2006). Also in this case, transcriptional activity of the tRNA gene was required for barrier function. However, a transcription-independent, but TFIIIC-dependent mode of barrier function has in addition been identified in both fission and budding yeast. It relies on the assembly of partial Pol III pre-initiation complexes that do not result in transcription of associated genomic sequences. These sites, referred to as 'extra TFIIIC' sites (ETC; Moqtaderi and Struhl 2004) in budding yeast or 'chromosome organizing clamps' (COC; Noma et al. 2006) in fission yeast recruit TFIIIC only or TFIIIC and TFIIIB (Noma et al. 2006; Simms et al. 2008; Valenzuela et al. 2009). Such ETCs seem to be evolutionarily conserved and have likewise been identified by ChIP-Seq experiments in human cells (Moqtaderi et al. 2010; Oler et al. 2010), often being located close to CTCF binding sites.

12.6 Alu-SINEs

Small interspersed nuclear elements (SINEs) belong to transposable elements (TEs). TEs can be subdivided into DNA transposons and retrotransposons. Retrotransposons can be further distinguished into long terminal repeat (LTR)-containing and non-LTR retrotransposons, the latter of which include Alu SINEs. The about one million Alu elements in humans collectively account for ~11% of the genome (International human genome sequencing consortium 2001; Cordaux and Batzer 2009). Alu RNAs represent fusion products of left and right monomers, each of which have been derived from the SRP (7SL) RNA. The left arm of Alu elements carries an internal type 2 promoter composed of an A- and a B Box that, together with 5′ and 3′-flanking sequences, directs transcription (Shaikh et al. 1997; Alemán et al. 2000; Berger and Strub 2011). Alu RNA has been shown to be involved in the regulation of Pol II transcription, alternative splicing, mRNA stability and of translation (reviewed in Häsler et al. 2007; Ponicsan et al. 2010; Berger and Strub 2011). Under normal physiologic conditions, most Alu elements are thought to be transcriptionally silent (Liu et al. 1994; Schmid 1998). Repression of Alu transcription is conferred, at least in parts, by the presence of positioned nucleosomes over the transcribed region (Englander et al. 1993; Englander and Howard 1995; Russanova et al. 1995; Tanaka et al. 2010). Various cellular stresses activate Alu transcription, possibly due to alterations in chromatin structure at these sites (Russanova et al. 1995; Li et al. 2000; Kim et al. 2001). DNA methylation is also involved in the repression of Alu element transcription and treatment with 5-azacytidine coincides with hypomethylation of these elements and increased abundance of Alu RNA (Liu and Schmid 1993; Liu et al. 1994).

Several genetic diseases have been linked to Alu elements. Retrotransposition of transcriptionally active young Alu elements into introns or exons of regulatory genes has been shown to be the underlying cause of diseases like hemophilia A

(factor VIII) and B (factor IX), neuofibromatosis (NF1), Huntington disease (ADD1), Apert syndrome (FGFR2) or also breast cancer (BRCA2) (reviewed in Ostertag et al. 2003; Callinan and Batzer 2006). In addition to Alu retrotransposition-mediated diseases, there are other examples that link Alu elements as the source of microsatellites to disease development. Alu element-dependent expansion of a GAA triplet repeat within the first intron of the frataxin gene has been associated with Friedreich ataxia (Justice et al. 2001; Clark et al. 2004). In addition, Spinocerebellar ataxia type 10 was shown to be caused by the Alu-mediated acquisition of unstable pentanucleotide repeats within intron 9 of the human ataxin 10 (ATXN10) gene (Matsuura et al. 2000; Kurosaki et al. 2006, 2009). With respect to trinucleotide expansion diseases, it is noteworthy that CTCF, which colocalizes with Pol III promoter elements (Moqtaderi et al. 2010) has likewise been implicated in the origin of generating fragile sites within trinucleotide repeats (Libby et al. 2008). Alu elements are furthermore sites at which genomic duplications or recombination-mediated deletions occur, which have been shown to result in genetic diseases, including cancer (Deininger and Batzer 1999).

Alu elements do not only contribute to the modification of primate genomes by causing insertions or deletions, but they also affect the expression of nearby genes through intrinsic boundary element activity. The Alu2 element that flanks the human keratin 18 (K18) gene permits position-independent expression of the K18 gene (or of reporter genes) in transgenic mice. Transcription interference with neighbouring genes was dependent on Pol III activity, but boundary element insulator activity was not (Neznanov and Oshima 1993; Willoughby et al. 2000). In addition, a B2 SINE was implicated in the stage-specific activation of the growth hormone gene in mice. B2 SINEs are retrotransposons derived from tRNA genes. At embryonic stage 17.5, the chromatin structure at the GH promoter and at a site 10–14 kb upstream the transcription start site (TSS) changed from hetero- to euchromatin. The site 10–14 kb upstream the TSS was shown to contain a B2 element and both Pol III sense as well as Pol II antisense transcripts could be detected. Boundary activity was dependent on Pol II transcription only (Lunyak et al. 2007). Recently, it was shown that Pol II and Pol III transcription from the same DNA strand of an Alu-like mouse B1 SINE (B1-X35S) was required for insulator activity (Román et al. 2011). Interestingly, the B1-X35S was bound by CTCF which has also been shown to colocalize with cohesin (Nasmyth and Haering 2009). As mentioned before, cohesin is found at human Alu elements, together with the ISWI chromatin remodelling complex (Hakimi et al. 2002; Fig. 12.3).

12.7 Perinucleolar Compartments (PNCs)

The perinucleolar compartment (PNC) is a dynamic structure that is localized close to the nucleolus (Matera et al. 1995). Its formation as a visible subnuclear body has been correlated with cell transformation and particularly with the capacity of cells to form metastases. The PNC is enriched in RNA-binding proteins, such as CUG-BP,

KSRP, nucleolin, PTB, raver 1/2 and ROD1, which contribute to RNA processing and splicing. In addition to these proteins, Pol III-transcribed RNAs, including RNAse P RNA, RNAse MRP RNA, SRP (7SL) RNA, hY RNAs and Alu RNAs have been identified in PNCs (Pollock and Huang 2010). PNCs seem to be associated with DNA, since treatment of cells with histone deacetylase inhibitors alters the morphology of PNCs and since DNA replication uncoupled from cell division increases the number of PNCs (Norton et al. 2009). However, the DNA loci do not contain Pol III genes. Nevertheless, the establishment of PNCs is dependent on ongoing Pol III transcription, which can partially be replaced by expression of RNAse MRP RNA from an ectopic Pol II promoter (Wang et al. 2003). Thus, Pol III RNAs are involved in the formation of a discrete subnuclear structure, the PNC. Analysis of more than 50 non-transformed to fully transformed cell lines derived from mouse and human stromal, endothelial or hematopoietic cells and also from embryonic stem cells showed that PNCs specifically form in cells from solid tumor tissues (Norton et al. 2008) and that their prevalence reaches near 100% in metastatic breast cancer (Kamath et al. 2005). Interestingly, PNCs were not detectable in mouse or human WA07 embryonic stem cells, but in murine F9 teratocarcinoma cells. The identification of an isoform of human RNA polymerase III (Pol IIIα) that is expressed in embryonic H1 stem cells and in cells transformed by defined genetic elements (Haurie et al. 2010) suggests that this isoform alone cannot be responsible for the establishment of PNCs, but may well participate or even be essential for their formation.

12.8 Conclusions

RNA polymerase III transcription is regulated by chromatin structure. In turn, Pol III genes or promoter elements, together with their partial or complete transcription machinery, in the absence or presence of ongoing transcription and depending on the genomic context, contribute to nuclear chromatin organization and the separation of chromatin domains. Thus, Pol III transcription and regulation of chromatin accessibility are interwoven by multiple mechanisms which may be linked to each other by feedback-loops, where chromatin accessibility allows for Pol III transcription, permitting the establishment of chromatin barriers, leading to nucleosome-free regions that, as a consequence, permit transcription and so on. The exciting finding that Pol III genes are occupied by proteins, such as cohesin, condensin or CTCF that contribute to establishing higher order chromatin structures leads to the assumption that the Pol III transcription machinery may contribute to cellular homeostasis beyond the traditionally appreciated role in the production of essential RNAs. In particular, the interaction of ETCs with CTCF or of cohesin with Alu elements, as well as the knowledge of cell type-specific interactions of CTCF with DNA and of cell type-specific methylation of Alu elements raises the possibility that Pol III-dependent establishment of higher chromatin structures may contribute to the regulation of gene expression by Pol II in a tissue- or cell type-specific manner.

Future experiments will show to which extent novel, chromatin-associated functions of Pol III transcription are important for the regulation of developmental processes, of cellular differentiation or, more in general, of health and disease.

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Chapter 13
The Role of DNA Methylation and Histone Modifications in Transcriptional Regulation in Humans

Jaime L. Miller and Patrick A. Grant

Abstract Although the field of genetics has grown by leaps and bounds within the last decade due to the completion and availability of the human genome sequence, transcriptional regulation still cannot be explained solely by an individual's DNA sequence. Complex coordination and communication between a plethora of well-conserved chromatin modifying factors are essential for all organisms. Regulation of gene expression depends on histone post translational modifications (HPTMs), DNA methylation, histone variants, remodeling enzymes, and effector proteins that influence the structure and function of chromatin, which affects a broad spectrum of cellular processes such as DNA repair, DNA replication, growth, and proliferation. If mutated or deleted, many of these factors can result in human disease at the level of transcriptional regulation. The common goal of recent studies is to understand disease states at the stage of altered gene expression. Utilizing information gained from new high-throughput techniques and analyses will aid biomedical research in the development of treatments that work at one of the most basic levels of gene expression, chromatin. This chapter will discuss the effects of and mechanism by which histone modifications and DNA methylation affect transcriptional regulation.

13.1 DNA Methylation

13.1.1 CpG Islands

With respect to epigenetic research and a causal relationship to human disease, DNA methylation is the most characterized modification. The enzymatic addition of a methyl group to DNA is performed by DNA methyltransferase (DNMT) on the

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5′-carbon of the pyrimidine ring in cytosine. Four human DNMTs have been characterized: DNMT1 (Bestor et al. 1988), DNMT2 (Yoder and Bestor 1998), DNMT3a and DNMT3b (Okano et al. 1999). *De novo* DNA methylation patterns are established early in development by DNMT3a and DNMT3b and maintained by DNMT1, which prefers to methylate hemi-methylated templates during DNA replication through its recruitment by proliferating cell nuclear antigen (PCNA). Around 3% of cytosines are methylated in the human genome almost exclusively in the context of the dinucleotide, CpG. 5-methylcytosine (5-mC) is also found in very low abundance at the trinucleotide, CpNpG (Clark et al. 1995; Lee et al. 2010).

CpG dinucleotides are rarer than expected in the human genome (~1%) (Josse et al. 1961; Swartz et al. 1962) as a result of 5-mC deamination and subsequent mutation to thymine (Scarano et al. 1967). 70–80% of CpG dinucleotides are methylated and those dinucleotides that are unmethylated tend to cluster in islands (Ehrlich et al. 1982). Regions containing the normal expected density of CpG dinucleotides are called CpG islands (CGI), which are regions no smaller than 200 bp that contain a GC content of more than 55% and an expected GC content to observed GC content ratio greater than 0.65 (Takai and Jones 2002).

Approximately 60% of human gene promoters and first exons are associated with CGIs (Bird 2002). CGIs at promoters are frequently hypomethylated corresponding to a permissive chromatin structure in order to poise genes for a transcriptional activation (Larsen et al. 1992; Antequera and Bird 1993) while some are hypermethylated during development, which stably silences the promoter (Fig. 13.1a) (Straussman et al. 2009). Such programmed CGI methylation is important for genomic imprinting, which results in monoallelic expression through the silencing of a parental allele (Kacem and Feil 2009) and gene dosage compensation such as X-chromosome inactivation in females (Reik and Lewis 2005). Recently, Doi et al. has shown that limited gene expression in differing tissue types is caused by differential methylation of CpG island shores (2009), which are located within 2.0 kb of CGIs (Fig. 13.1b) (Saxonov et al. 2006). Still, a fraction of CGIs are prone to methylation in some tissues due to aging, in promoters of tumor suppressor genes in cancer cells (Issa 2000) and committed cell lines (Jones et al. 1990). The remaining 40% of CGIs are located intra- and intergenically. Intragenically located CGIs within the coding region of genes are methylated at trinucleotides CpXpG (Lister et al. 2009) and are commonly found in highly expressed, constitutively active genes (Fig. 13.1c) (Zhang et al. 2006) while intergenic CGIs may be used for transcription of non-coding RNAs (Illingworth et al. 2008).

13.1.2 Transcriptional Regulation

More often than not, DNA methylation is usually associated with gene silencing due to (1) the occlusion of DNA binding proteins that act as or recruit transcriptional activators or (2) the recruitment of methyl-binding proteins (MBPs), which recruit transcriptional corepressor complexes (Fig. 13.1a). Transcriptional activators and

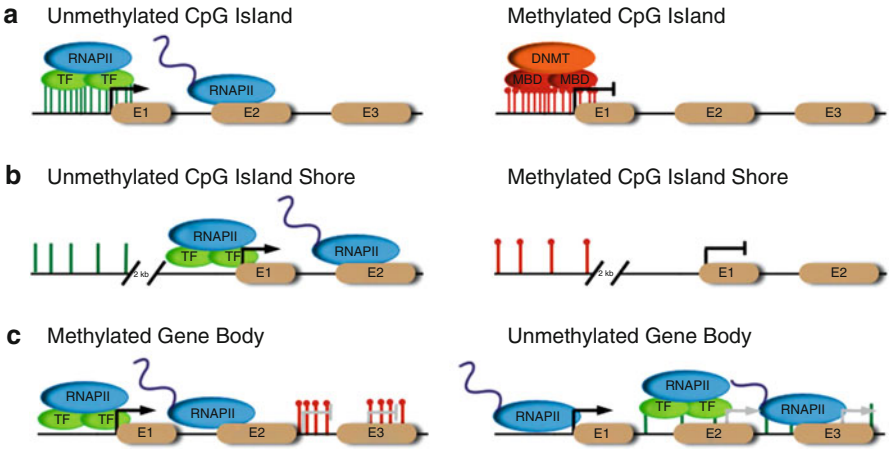

Fig. 13.1 Various sites and effects of DNA methylation throughout the genome. DNA methylation is found at inter- and intragenic regions throughout the genome. DNA methylation dependent transcriptional activity is contingent on CpG dinucleotide genic location and density. Normal methylation events and subsequent effects are shown on the left. (**a**) CpG islands at promoters are normally unmethylated resulting in gene expression. However, aberrant hypermethylation at the same promoter results in corepressor complex recruitment and subsequent gene repression. (**b**) Intragenic regions characterized by scattered CpG dinucleotides located 2 kb upstream of the promoter called CpG island shores are regulated in the same manner as (**a**). (**c**) DNA methylation within the gene body prevents initiation of transcription from spurious sites in the gene. If unmethylated, these sites become transcriptional start sites resulting in an incorrect product (Portela and Esteller 2010)

repressors recruit histone modifying and chromatin remodeling complexes that can remodel chromatin, which ultimately changes the transcriptional activity of a gene. Modifications made by such complexes and subsequent effects on transcription will be discussed later.

Even previous to DNA methylation, DNMTs can be recruited to DNA via DNA binding transcription factors, which results in specific promoter DNA methylation and regulatory gene repression. For example, studies (Di Croce et al. 2002) showed that DNMTs interact with the oncogenic transcription factor formed by the fusion of promyelocytic leukemia protein and retinoic acid receptor (PML-RAR), found in acute promyelocytic leukemia. DNMT recruitment to the *RARβ2* gene promoter by PML-RAR results in promoter hypermethylation and subsequent gene silencing (Di Croce et al. 2002). A similar mechanism has been described for Myc, a DNA binding transcription factor. Myc interacts with DNMT3a and is recruited to the p21 gene promoter resulting in subsequent DNA methylation and p21 gene repression (Brenner et al. 2005). In addition, p53 also interacts with DNMT3a and represses p53′s transactivator function at the p21 gene promoter but in a DNA methylation independent manner (Wang et al. 2005). Both mechanisms elucidate cancer promoting pathways that intersect with DNA methylation and cause repression of p21 expression, a cyclin dependent kinase inhibitor. Moreover, one study (Hervouet et al. 2009)

showed that DNMT3a/b interacts with 79 different DNA binding transcription factors. Some interactions were exclusive to each DNMT while some were shared between both (Hervouet et al. 2009). The diversity of interactions further illustrates the importance of DNA methylation on gene expression regulation through DNMT recruitment via DNA binding transcription factors.

Once DNA is methylated, DNA methyl-binding proteins (MBP) can bind to DNA and recruit transcriptional corepressors such as histone deacetylase (HDAC) complexes, polycomb proteins, and chromatin remodeling complexes. One family consists of MBPs, which possess a conserved methyl-CpG-binding-domain (MBD) and includes MBD1, MBD2, MBD3, MBD4, and MeCP2. MeCP2 is the founding member of the MBD family and contains a MBD in addition to an adjacent transcriptional repressor domain (TRD) (Klose and Bird 2006). The TRD interacts with Sin3 corepressor complex containing HDAC1 and 2 (Nan et al. 1998). MBD1 also contains three zinc-binding domains (CxxC), which has been shown to be responsible for its ability to bind unmethylated CpG sites (Jorgensen et al. 2004). MBD1 and 2 both contain a TRD that recruits different transcriptional corepressor complexes containing HDACs. MBD3 contains a MBD but does not bind methylated DNA due to two amino acid substitutions (Hendrich and Tweedie 2003) but is associated with the nucleosome remodeling and histone deacetylase (NuRD) corepressor complex, which contains HDACs necessary for transcriptional silencing. MBD4 is a thymidine glycosylase DNA repair enzyme that excises mismatched thymines that have resulted from 5-methylcytosine deamination in the context of CpG dinucleotides (Hendrich et al. 1999).

The second family of MBPs includes Kaiso, zinc finger and BTB (for BR-C, ttk, and bab) domain containing (ZBTB) 4 and ZBTB38 (Zollman et al. 1994). These are atypical MBPs, because they depend on a zinc-finger domain to recognize methylated DNA and a POZ (for Pox virus and Zinc finger) (Bardwell and Treisman 1994)/BTB domain to repress transcription through its interaction with nuclear receptor co-repressor-1 (N-CoR) (Prokhortchouk and Defossez 2008). Another study (Iioka et al. 2009) showed that Kaiso can regulate transcription factor activity by modulating the interaction between β-catenin and HDAC1 activity. The third family of MBPs includes ubiquitin-like plant homeodomain and RING finger (UHRF)-domain containing protein 1 and 2. Both contain SET and RING associated (SRA) domains, which preferentially bind to DNMT1's substrate, hemi-methylated DNA (Bostick et al. 2007). Furthermore, UHRF1 has been shown to colocalize with DMNT1, which suggests that this family of MBPs may help target DMNT1 to DNA (Bostick et al. 2007).

DNA methylation is usually associated with transcriptional silencing, and one of the most well known cases where differential DNA methylation induces and suppresses expression is genomic imprinting at the *H19/IGF2* locus. Genomic imprinting is a form of gene regulation in which an allele is expressed from one of the two parental homologous genes. *H19* and *IGF2* are reciprocally imprinted so that *H19* is expressed from the maternally inherited allele and *IGF2* from the paternally inherited allele (Bell and Felsenfeld 2000). Transcriptional regulation of these genes is dependent on a differentially methylated DNA domain (DMD) or

imprinting control region (ICR) located upstream of *H19* and downstream of *IGF2*. The DMD/ICR is methylated on the paternal allele but not the maternal allele (Bell and Felsenfeld 2000; Hark et al. 2000; Szabo et al. 2000; Kanduri et al. 2000). CCCTC-binding factor (CTCF) binds to the unmethylated ICR of the maternal allele, which blocks an enhancer region located downstream of *H19* from activating transcription of *IGF2* (Hark et al. 2000). CTCF binding also protects against *de novo* methylation and subsequent repression at the *H19* locus on the maternal allele (Rand et al. 2004). This is one of the most basic examples of how differentially methylated regions can determine levels of gene expression. Mutations or deletions in the *H19* promoter, ICR, or enhancer can lead to growth defects such as Beckwith-Wiedemann Syndrome or Silver-Russell dwarfism (Delaval et al. 2006).

With the advent of microarrays and high-throughput technologies, an explosion of gene expression profile comparisons in normal and diseased cells has occurred. Many studies have pursued genes of interest by comparing the DNA methylation status of a gene's 5′ promoter region (Weber et al. 2005; Hatada et al. 2006), and presently, more comprehensive results are available as more direct solutions to discovering gene expression controlled by DNA methylation are established. Using *Arabidopsis thaliana* as a model system, Zhang et al. analyzed and compared whole genome methylome tiling arrays gathered from immunoprecipitating 5-mC or chromatin crosslinked MBPs in normal and mutant cells (Zhang et al. 2006). Another study (Javierre et al. 2010) compared the DNA methylome of monozygotic twins who were differently affected by the disease, systemic lupus erythematosus (SLE) (Javierre et al. 2010). In comparison to the healthy twin, the twin affected by SLE had a decrease in promoter DNA methylation for many genes involved in immune system function including *IFNGR2, MMP14, LCN2, CSF3R, PECAM1, CD9, AIM2,* and *PDX1*. These genes had also previously been shown to participate in the development of SLE (Javierre et al. 2010).

13.1.3 5-Hydroxymethylcytosine

In the previous sections, 5-methylcytosine (5-mC) was discussed extensively. 5-mC can be converted to 5-hydroxymethylcytosine (5-hmC) by an oxidation reaction carried out the ten-eleven-translocation (TET) family of proteins (Tahiliani et al. 2009). 5-hmC was first discovered in bacteriophage DNA in 1952 (Wyatt and Cohen 1952; Warren 1980) and has since been found to be enriched in mouse brain (Kriaucionis and Heintz 2009), embryonic stem cells (Tahiliani et al. 2009), and human tissues (Li and Liu 2011).

Levels of 5-hmC are dynamically regulated by TET1-3 in stem cells and seem to be higher in pluripotent cells. Knockdown of *TET1* and *TET2* causes a decrease in 5-hmC levels and an increase in 5-mC at stem cell related gene promoters (Ficz et al. 2011). These genes are subsequently silenced. *TET3* is highly expressed in zygotes and oocytes (Wossidlo et al. 2011) and a recent study (Iqbal et al. 2011) has shown that after fertilization, 5-mC is converted to 5-hmC in the male but not

the female pronucleus. This data (Iqbal et al. 2011) suggests an alternative to the global demethylation theory during cellular dedifferentiation where genome-wide 5-mC may be converted to 5-hmC by TET3 and differentiation is promoted by a decrease in TET3 and an increase in TET1 and 2 (Koh et al. 2011; Walter 2011). The mechanisms behind 5-hmC's role in cellular differentiation (Ito et al. 2010), carcinogenesis, (Li and Liu 2011) and association with actively transcribed genes is a mystery (Ficz et al. 2011). One clue provided is that 5-hmC prevents the binding of MBDs (Valinluck et al. 2004) and DNMTs (Valinluck and Sowers 2007).

13.2 Histone Modifications

13.2.1 Types of Modifications

As mentioned in the previous section, methylated DNA can recruit different transcriptional activator and repressor complexes. In most cases, these complexes contain histone modifying and chromatin remodeling enzymes that regulate chromatin structure, which ultimately changes the transcriptional activity of a gene. Such complexes are not just recruited by DNA methylation but also by various post-translational modifications (PTMs) of the proteins that make up chromatin. In this section, the effects of histone modifications and chromatin remodeling on gene expression will be discussed.

Chromatin is the organization of the eukaryotic genome into a condensed form due the function of many proteins and RNAs. The fundamental unit of the highly ordered chromatic fiber is the nucleosome, which consists of 146 base pairs of DNA wrapped around an octamer of core histones that contains two of each histone H2A, H2B, H3, and H4. A linker histone, H1, binds to DNA as it enters and exits its 1.65 turns around the nucleosome (Luger et al. 1997). Naturally, the condensed structure forms a barrier to cell processes that require accessibility to DNA such as DNA replication, damage repair, and transcription (Workman and Kingston 1998). Covalent HPTMs such as acetylation, methylation, phosphorylation, ubiquitination, SUMOylation, ADP-ribosylation, deimination, and the non-covalent proline isomerization (Kouzarides 2007) can affect the condensation of chromatin as to organize the genome into transcriptionally active and inactive regions termed euchromatin and heterochromatin (Heitz 1929) respectively and recruit effector proteins (Jenuwein 2001).

The core histones are highly conserved basic proteins composed of a globular domain and highly flexible N-terminal tails that protrude from the DNA wrapped nucleosome (Luger et al. 1997). All histone N-terminal tails and globular domains are subject to modification and more is known about the smaller covalent modifications methylation, acetylation, and phosphorylation. Lysine residues can be mono-, di-, and trimethylated while arginine residues can only be mono- or symmetrically or asymmetrically dimethylated (Bannister and Kouzarides 2011). The interactions between chromatin associated proteins that bind HPTMs can act

Table 13.1 Transcriptional and cellular role of histone modifications

Modification	Histone residues modified	Role in cell activity and transcription	Histone modification readers
Acetylated lysine (Kac)	H3 (K4,9,14,18,23, 27,36,56)	Activation	Bromodomain
	H4 (K5,8,12,16,19)		
	H2A (K5,9)	DNA damage repair	Tandem PHD
	H2B (K5,6,7,12,16, 17,20,120)		
Phosphorylated serine/	H3 (S10,28 and T3,11,45)	Apoptosis	14-3-3 Domain
	H4 (S1,47)	Activation	
Threonine (S/Tph)	H2A (S1)	Mitosis (Baker et al. 2010)	
Methylated lysine (Kme)	H3 (K4,23,36,79)	Activation	MBT PHD
	H3 (K9, 27) and H4 (K20)	Repression	Tudor Chromodomain WD40
Methylated arginine (Rme)	H3 (R2,17,26) and H4 (R3)	Activation	Tudor (Yang et al. 2010; Chen et al. 2011)
	H3 (R8)	Repression	ADD (Zhao et al. 2009)
Ubiquitylated lysine (Kub)	H2A (K119)	Repression	Cps35 (Lee et al. 2007a; Zheng et al. 2010)
	H2B (K120)	Activation (Zhu et al. 2005)	
Sumoylated lysine (Ksu)	H4 (?)	Repression (Shiio and Eisenman 2003)	

synergistically or antagonistically with one another resulting in various gradients of transcriptional activation and repression across the genome. The term "histone code" was coined in order to convey that chromatin modifying proteins ultimately determine phenotype rather than simple possession of a certain genetic code (Strahl and Allis 2000; Jenuwein and Allis 2001). HPTMs specific roles in gene expression and cellular activities are shown in Table 13.1 (adapted from Kouzarides and Berger 2007; Wang et al. 2008).

Euchromatin is characterized by high levels of acetylation and high levels of H3K4me1/2/3, H3K36me3 and H3K79me1/2/3. On the other hand, heterochromatin is characterized by low levels of acetylation and high levels of H3K9me2/3, H3K27me2/3 and H4K20me3 (Table 13.1) (Li et al. 2007a). More recently, a group (Wang et al. 2008) performed chromatin immunoprecipitation sequencing (ChIP-seq) on 39 different core histone acetylations and methylations at 3,286 promoter regions. As shown in previous studies (Turner et al. 1992), acetylated histones consistently correlate with increased gene transcription. However, certain modifications localized to specific gene regions rather than just at transcriptional start sites (TSS). H2AK9ac, H2BK5ac, H3K9ac, H3K18ac, H3K27ac, H3K36ac and H4K91ac were mainly located in the region surrounding the TSS, whereas

H2BK12ac, H2BK20ac, H2BK120ac, H3K4ac, H4K5ac, H4K8ac, H4K12ac and H4K16ac were prominent in the promoter and transcribed regions of active genes (Wang et al. 2008).

Another group (Karlic et al. 2010) analyzed ChIP-seq data produced by the Zhao lab in order to create a model that could predict levels of gene expression based on HPTM levels present at promoters. They found that actively transcribed genes are characterized by high levels of H3K4me3, H3K27ac, H2BK5ac and H4K20me1 in the promoter and H3K79me1 and H4K20me1 along the gene body. Moreover, they found high levels of H4K20me1 and H3K27ac at promoters that contained high CpG content and H3K4me3 and H3K79me1 at promoters with low CpG content (Karlic et al. 2010). Although there is no model that explains the HPTM difference at the two types of promoters, one can guess that the difference is caused by different regulatory mechanisms and possibly, changes in DNA methylation. In agreement with this theory, a recent paper (Ernst and Kellis 2010) used previous ChIP-seq data for HPTMs, CTCF, RNA Polymerase II (RNAPII), and the histone variant, H2A.Z, to describe 51 distinct chromatin states. Each state is described by the enrichment of different HPTMs and chromatin associated proteins across the genome. Moreover, biological states of cells (cell cycle, developmental, T-cell activation, *etc.*) were predicted using the 51 epigenetic states (Ernst and Kellis 2010). Another interesting study (Mikkelsen et al. 2007) showed that embryonic stem cells contained a bivalent pattern of HPTMs at promoters of genes that regulate development. Surprisingly, they found H3K4me3, an activation mark, and H3K27me3, a repressive mark, co-localizing at these promoters in stem cells (Mikkelsen et al. 2007). These bivalent domains can resolve into four different chromatin states: (1) marked with both H3K4me3 and H3K27me3; (2) marked with neither H3K4me3 nor H3K27me3; (3) marked with H3K4me3 alone; (4) marked with H3K27me3 alone. Maintenance or loss of both marks results in a poised transcriptional state while preservation of H3K27me3 alone or H3K4me3 alone results in inactive and active transcription respectively (Cui et al. 2009). This data (Mikkelsen et al. 2007) suggests that HPTM bivalency at promoters allows for plasticity during cellular differentiation and development (Bernstein et al. 2006).

13.2.2 Transcriptional Regulation

As presented in the previous section, various HPTMs correlate with gene expression and repression (Fig. 13.2). Until recently, elucidating the mechanisms by which HPTMs interact with one another to control transcriptional activity has been complicated due to the layered complexity of combinatorial HPTMs and HPTM crosstalk. However, analysis of recently acquired ChIP-seq data and associated gene expression profiles has speedily facilitated decipherment of the histone code and its effect on transcriptional activity (Figs. 13.2 and 13.3) (Barski et al. 2007; Wang et al. 2008; Heintzman et al. 2007; Mikkelsen et al. 2007). Three broad effects on transcription can be attributed to HPTMs: (1) HPTMs can prevent certain chromatin

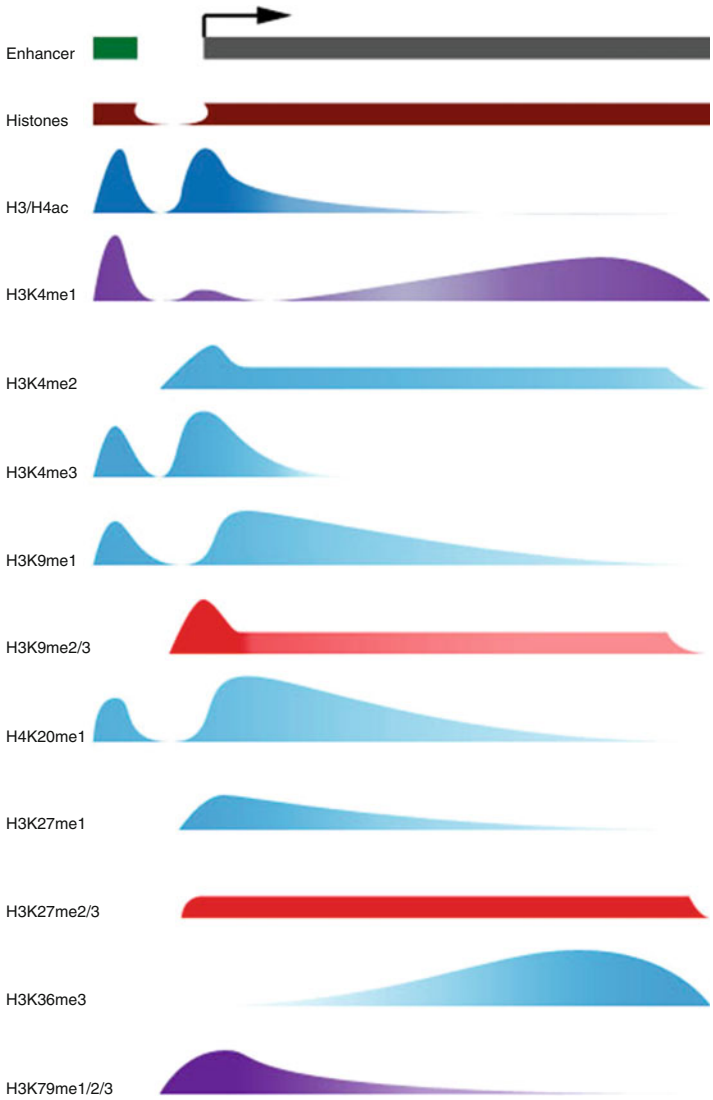

Fig. 13.2 Localization of histone modifications across genes as it relates to transcriptional regulation. Patterns of histone modification enrichment are shown across an arbitrary enhancer and gene. The enhancer is shown as the smaller region succeeded by a gap denoting a nucleosome-free region and transcriptional start site as shown by the *arrow*. Data used to compile the profiles are from GWAS on histone modifications. The correlative effects of the modifications on gene expression are indicated by different colors: *blue* for expression, *red* for repression, and *purple* for enrichment of the mark in both repressed and expressed genes (Wang et al. 2008; Barski et al. 2007; Li et al. 2007a)

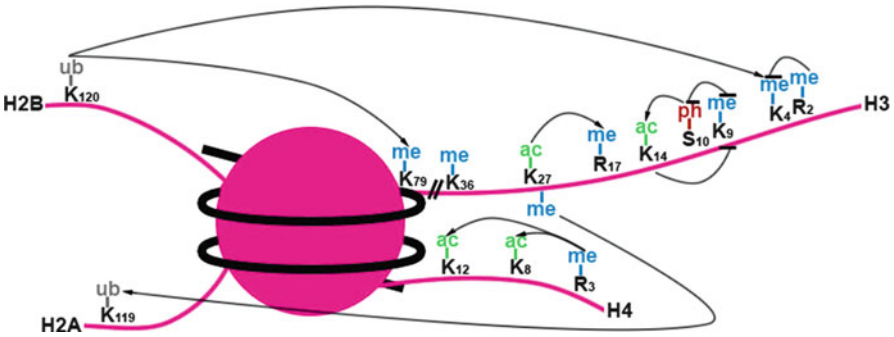

Fig. 13.3 Histone modification crosstalk. Various post-translational histone modifications affect the binding of certain domains and catalysis of other HPTMs. *Arrows* indicate a positive effect and *bars* indicate inhibitory effects on other HPTMs (Bannister and Kouzarides 2011)

binding proteins from binding. For example, H3S10ph prevents heterochromatin protein 1 (HP1) from binding H3K9me3 (Kouzarides and Berger 2007); (2) HPTMs can recruit certain chromatin binding proteins, which can enhance or inhibit gene activation. For example, H3K9me3, a marker for mammalian heterochromatin, is bound by the chromodomain of HP1 resulting in chromatin condensation and occlusion of DNA and nucleosomal binding sites utilized by coactivators, transcription factors, and RNAPII (Kouzarides and Berger 2007); (3) HPTMs can act *in cis* by affecting transcription through alteration of chromatin structure. For example, H4K16ac alone prevents the formation of a higher ordered compacted chromatin structure resulting in chromatin decondensation and increased transcriptional activity (Shogren-Knaak et al. 2006).

The following sections will focus on the effects of histone (de)acetylation and methylation on gene expression. It should be noted that much of the mechanistic research done on transcriptional regulation and HPTMs is pioneered through the use of yeast model systems because genetic manipulation and high-yield results have been easier to obtain as compared to humans. Importantly, many yeast proteins have correlative homologs that serve in the same manner as they do in mammals. However, there are some differences between the two eukaryotic organisms. For example, yeast do not possess the repressive mark H3K27me and in some cases, homologous complexes may contain different chromatin targeting proteins.

13.2.3 Histone Acetylation and Deacetylation

Histone acetylation at conserved lysine residues is the most intensely studied HPTM and was the first modification linked to transcriptional activity (Hebbes et al. 1988). It was not until 1996, that a direct molecular link was made between acetylation and

transcription. The first nuclear histone acetyltransferase (HAT) discovered, p55, was orthologous to a previously isolated transcriptional coactivator in *Saccharomyces cerevisiae*, Gcn5 (Brownell et al. 1996). HATs catalyze the addition of acetyl-coA to the ε-amino group on lysine side chains resulting in charge neutralization and affinity reduction between negatively charged DNA and basic histones. Acetylation ultimately creates an "open" chromatin structure (Shogren-Knaak et al. 2006) poised for active transcription through exposure of DNA-binding sites (Vettese-Dadey et al. 1996). There are two types of HATs: type-A (nuclear) and type-B (cytoplasmic). This discussion will only focus on type-A as they catalyze reactions related to active transcription (Bannister and Kouzarides 2011).

Type-A HATs are further divided into five families including the GCN5-related *N*-acetyltransferases (GNATs); the MOZ, Ybf2/Sas3, Sas2 and Tip60 (MYST)-related HATs; p300/CREB-binding protein (CBP) HATs; the general transcription factor HATs including the TFIID subunit TBP-associated factor-1 (TAF1); and the nuclear hormone-related HATs SRC1 and ACTR (SRC3) (Nagy and Tora 2007). They are often part of larger protein complexes and are recruited by DNA binding activators. For instance, in yeast, Gcn5 is part of the Spt-Ada-Gcn5-Acetyltransferase (SAGA) and Adaptor (ADA) complexes (Grant et al. 1997). In SAGA, Gcn5 is associated with three protein families known to be involved in gene expression: Spt, Ada, and a subset of TAFs (Grant et al. 1998). SAGA is recruited to active promoters via the SAGA subunit, Tra1's interaction with acidic activator domains of transcriptional activators and subsequent recruitment of the TATA-binding protein (TBP) by the subunit, Spt3 (Grant et al. 1998; Larschan and Winston 2001; Brown et al. 2001; Reeves and Hahn 2005). Similar complex subunits have been found to be associated with Gcn5 human homologs, p300/CBP associated factor (P/CAF) and hGcn5 (Ogryzko et al. 1998; Martinez et al. 1998; Nagy and Tora 2007). Human Gcn5 is found in the SAGA complex homolog Spt3-Taf9-Gcn5-Acetyltransferase (STAGA) complex and is recruited to promoters by the Tra1 human homolog, Transactivation/transformation domain associated protein (TRRAP) via its interaction with the transactivation domain of c-Myc (McMahon et al. 2000; Liu et al. 2003).

Furthermore, Gcn5, P/CAF, and p300 contain a bromodomain that bind acetyl-lysine. Taf1 contains two bromodomains (Jacobson et al. 2000). The exact function of bromodomains has yet to be elucidated. However, it is speculated that once HAT complexes are targeted to the promoter and perform acetylation, subsequent coactivators can stably bind to acetylated histone rich promoter regions via bromodomains, which would facilitate an acetylation cascade. Consistent with this hypothesis, SAGA requires the functional bromodomains of Gcn5 and the remodeling complex proteins Swi2/Snf2 for stable promoter occupancy, efficient HAT activity, and increase in gene expression resulting from an "open" chromatin conformation, and subsequent gene activation (Hassan et al. 2002). It should also be noted that HATs also acetylate non-histone proteins including the tumor suppressor p53 and various transcription factors (Glozak et al. 2005), which ultimately regulates gene expression (Sterner and Berger 2000).

Like many bromodomains, DPF3b, a novel acetyl-lysine reader and BAF remodeling complex associated subunit also binds ambiguously to acetylated H3 and H4

(Lange et al. 2008) via its tandem plant homeodomain (PHD) fingers (Zeng et al. 2010). One PHD finger only has affinity for acetylated H3K14, which increases full-length DPF3b's affinity for acetylated H3 and H4 (Zeng et al. 2010). Loss of DPF3b affects both skeletal and heart muscle development through transcriptional deregulation of other transcriptional factors (Lange et al. 2008).

On the other hand, histone deacetylases (HDACs) reverse the reaction carried out by HATs by removing acetyl marks on lysine to restore the positive charge. They fall into four classes: Class I (HDAC1, 2, 3, and 8), II (HDAC4, 5, 6, 7, 9, 10), III or Sir2-related enzymes, and class IV, which contains one member, HDAC11. Class III HDACs require the cofactor NAD^+ for its activity (Yang and Seto 2007). HDAC1 and 2 are found in the mammalian complexes Sin3A/B, NuRD, and corepressor for RE1 silencing transcription factor/neural-restrictive silencing factor (CoREST) while HDAC3 is found in nuclear receptor corepressor/silencing mediator for retinoid and thyroid hormone receptors (N-CoR/SMRT) (Yang and Seto 2008). Some of these corepressor complexes contain methyl-lysine binders that help target complexes to specific site on chromatin. For instance, a subunit of the Sin3a complex, ING2, contains a PHD finger domain that binds H3K4me3 (Champagne and Kutateladze 2009) in response to DNA damage. Once Sin3a is recruited, HDAC1 activity is stimulated, which stabilizes nucleosomes resulting in the repression of cell proliferation genes as a response to genotoxic events (Shi et al. 2006).

13.2.4 Histone Methylation

Histone methylation is performed on the residues lysine and arginine by histone methyltransferase (HMT) enzymes. Lysines can be mono-, di-, and trimethylated while arginines can be mono- and symmetrically or asymmetrically dimethylated. There are over 20 sites of methylation that have been identified on the core histones. Given all the possible combinations of histone methylation, it is one of the most complex HPTMs to study in a static model. The modifications most relevant to transcriptional regulation have been listed in Table 13.1 and a few of the most studied histone methylations will be discussed in this section. Fig. 13.2 summarizes the transcriptional effects and genomic enrichment of the HPTMs discussed below.

13.2.4.1 H3K4

H3K4 methylation is usually enriched at the enhancers and promoters of actively transcribed genes (Wang et al. 2008; Santos-Rosa et al. 2002). H3K4me1 is highly enriched at enhancers (Wang et al. 2005). H3K4me2 is commonly found in the body of active genes while H3K4me3 is largely observed at the 5′ ORF of genes (Pokholok et al. 2005). Methylation of H3K4 results from the recruitment of various H3K4 HMT enzymes by transcriptional machinery, specifically RNAPII. Once RNAPII is poised for active transcription through phosphorylation of serine-5 of

the carboxy-terminal domain (CTD) by TFIIH (Phatnani and Greenleaf 2006), the Set1 containing H3K4 HMT complex, COMPASS, is recruited by the PAF complex (Ng et al. 2003; Wood et al. 2003). RNAPII is released into an early elongating complex where H2BK120 (K123 in yeast) is ubiquitylated, which is required for further Set1 activity. Sometime during elongation, RNAPII is phosphorylated at serine-2 resulting in the release of Set1 (reviewed in Martin and Zhang 2005).

Furthermore PAF also interacts with chromodomain containing protein Chd1 (Simic et al. 2003). Proteins possessing methyl-binding domains, called chromodomains, are recruited to the H3K4me3 enriched promoter. SAGA also interacts with Chd1, which has two chromodomains, one which helps recruit SAGA to sites of H3K4me2/3 (Pray-Grant et al. 2005). As discussed earlier, SAGA recruitment results in an acetylation cascade that further promotes transcriptional activation. In humans, the HMT containing mixed-lineage-leukemia (MLL) complex is recruited by the H3K4me2 binding domain, WDR5. WDR5 interacts preferentially with H3K4me2 through its WD40-repeat domain (Wysocka et al. 2005). MLL can then convert H3K4me2 to H3K4me3.

13.2.4.2 H3K36

Unlike the 5′ localization of H3K4 methylation, H3K36 methylation is highly enriched in the coding region and 3′ ORF of genes (Kolasinska-Zwierz et al. 2009). As mentioned in the previous section, once the CTD of RNAPII is phosphorylated at Serine-2 by Ctk1 and Bur1 kinases (Keogh et al. 2003; Qiu et al. 2009), Set1 is released and chromatin is primed for transcriptional elongation through recruitment of Set2 (Xiao et al. 2003; Krogan et al. 2003). Set2 HMT catalyzes H3K36 methylation and specifically binds to phosphorylated Serine-2 of RNAPII's CTD (Hampsey and Reinberg 2003). This form of RNAPII is found in the transcribed regions of genes and the 3′ end of genes, which correlates with H3K36me2/3 localization (Xiao et al. 2003; Krogan et al. 2003; Hampsey and Reinberg 2003; Li et al. 2003). The passage of RNAPII during transcriptional elongation results in histone displacement and positioning behind RNAPII. These histones are hyperacetylated and subsequently methylated by Set2 (Hampsey and Reinberg 2003; Carrozza et al. 2005; Joshi and Struhl 2005; Keogh et al. 2005).

H3K36me2 is recognized by the chromodomain of Eaf3 and PHD finger of Rco1, which are subunits of the Rpd3S HDAC complex (Joshi and Struhl 2005; Govind et al. 2010). During transcriptional elongation, Rpd3S is recruited via the serine-2/serine-5-diphosphorylated CTD repeats followed by H3K36me2 binding by Eaf3 and Rco1 (Keogh et al. 2005; Govind et al. 2010). Once Eaf3 and Rci1 are recruited by H3K36me2, Rpd3 is transferred from the phosphorylated CTD to H3 where its HDAC activity creates a hypoacetylated environment within gene bodies and at the 3′ end. (Li et al. 2007b; Govind et al. 2010). Deletion of Rco1 or Eaf3 results in hyperacetylation of ORFs and the production of aberrant transcripts that are presumably initiated from cryptic promoters that are usually silenced by the Set2-Rpd3 pathway after RNAPII progression (Carrozza et al. 2005; Joshi and Struhl 2005; Keogh et al. 2005).

13.2.4.3 H3K79

Unlike the previously discussed HPTMs, H3K79 methylation occurs in the globular domain of H3 and within the core of the nucleosome. It is found within the coding regions of genes and is usually associated with active chromatin. H3K79 methylation is catalyzed by the HMT, Dot1. Dot1 is the first lysine HMT that has been identified that's lacks an identifiable SET domain (Feng et al. 2002). Dot1 is required to prevent the spread of HDACs into active chromosomal regions (van Leeuwen et al. 2002). There is no protein that links H3K79 methylation to transcriptional regulation. However, mammalian hDot1L has been implicated in mediating the leukemogenic fusion protein MLL (for mixed lineage leukemia)-AF10. It was found that hDot1L is recruited to MLL–AF10 target genes, such as *HOXA9*, through an interaction between hDot1L and AF10 (Okada et al. 2005). Upregulation of *HOXA9* expression results in defective hematopoiesis and leukemogenic transformation making regulation of H3K79 methylation a possible therapeutic target. Also, mammalian protein 53BP1 interacts with H3K79me3 through a tudor domain at sites of DNA damage (Huyen et al. 2004).

13.2.4.4 H3K27

In mammals, H3K27 methylation is a repressive mark catalyzed by the Polycomb Repressor Complex 2 (PRC2), which contains the SET-domain containing lysine HMT, Enhancer of Zester 2 (EZH2). H3K27me3 serves as a repressive mark at homeotic genes, the inactive X-chromosome, and imprinted genes while H3K27me1 is enriched at pericentric heterochromatin (Martin and Zhang 2005). PRC2 is made up of four core components: EZH2, embryonic ectoderm development (EED), suppressor of zeste 12 homolog (SUZ12), and histone-binding protein retinoblastoma-binding protein p48/46 (RbAp48/46). Both EED and SUZ12 are necessary for EZH2 HMT activity (Simon and Kingston 2009). EED contains repeats of WD40 domains that bind H3K27me3 and promote PRC2 propagation (Margueron et al. 2009) and SUZ12 contains C_2-H_2 zinc finger and VEFS domain. RbAp48/46 contains six WD40 domains and is a core histone binding subunit.

PCR2 also interacts with AEBP2, PCLs and JARID2. AEBP2 contains three zinc-fingers that may play a role in DNA binding (Kim et al. 2009a). PCL1, PCL2 and PCL3 (also known as PHF1, MTF2 and PHF19, respectively) contain a tudor domain and two PHD finger proteins, a PCL extended domain and a carboxy-terminal domain tail (Wang et al. 2004a). PCL proteins interact with PRC2 through EZH2, and to some extent through SUZ12 and the histone chaperones RbAp46/48 (Nekrasov et al. 2007). JARID2 is the founding member of the Jumonji family of proteins that catalyses the demethylation of histone proteins. However, it lacks demethylase activity. JARID2 contains JmjC and JmjN domains and two potential DNA binding domains, ARID and a zinc finger (Margueron and Reinberg 2011). The core components of PRC2 and its associated proteins discussed above are all necessary for EZH2 optimal function.

The targeting of PRC2 in *D. melanogaster* is a well understood mechanism compared to humans. In *D. melanogaster*, transcription factors, such as Pho and PhoL, bind to the Polycomb responsive element and recruit EZ of PRC2. Only now is the mammalian mechanism coming to light with the recent discovery of long non-coding RNA (lncRNA) dependent PRC2 recruitment. The lncRNA, *HOTAIR*, is transcribed from the HOXC locus, binds PRC2, and targets the complex to the HOXD locus where several genes are repressed (Rinn et al. 2007). Also, the lncRNA *Xist* and a short internal transcript *RepA* have been to shown target PRC2 to the inactivated female X-chromosome, which subsequently is repressed and enriched with H3K27me3. In contrast the lncRNA and antagonist to *Xist*, *Tsix*, also interacts with PRC2 suggesting an inhibitory mechanism to X-chromosome inactivation (Zhao et al. 2008).

13.2.4.5 H3K9

H3K9 methylation is one of the most intensely studied histone modifications to date. H3K9me1 is catalyzed by methyltransferases HMT1C/G9a or demethylases KDM3A/JMJD1A and KDM4D/JMJD2D (Shi and Whetstine 2007). The mark is enriched at the 5′ UTR and found minimally in non-genic regions (Barski et al. 2007; Rosenfeld et al. 2009). Although no function has been ascribed to H3K9me1, its proposed mechanism of action may be to act as an intermediary between gene activation and repression through rapid methylation or demethylation (Black and Whetstine 2011). Most studies have focused on H3K9me2/3 as a heterochromatin mark catalyzed by the lysine HMT SUV39H1/2 and recognized by the chromodomain of heterochromatin protein-1 (HP1), which dictates the compaction of heterochromatin. H3K9me2/3 is enriched in pericentromeric, subtelomeric, and gene desert regions (Rice et al. 2003). Gene deserts are megabase sized regions devoid of coding genes, and unlike H3K9me3, H3K9me2 is rarely found in at individual active or silenced genes (Rosenfeld et al. 2009). In support of H3K9me2′s function as a repressive mark, it has been shown to associate with Lamin B1, a protein localized to the nuclear periphery and part of the nuclear lamina, which is commonly associated with inactive genes. Lamin B1 associated regions are also devoid of the activating mark, H3K4me3, and RNAPII further suggesting H3K9me2 is most likely a repressive mark that facilitates separation of active and inactive genes through chromosomal localization within the nuclear architecture (Guelen et al. 2008).

H3K9me3 is commonly found at heterochromatin and repressed promoters, and unlike H3K9me2, H3K9me3 is also localized to centromeres, subtelomeric regions, and in some cases, the coding region of genes (Vakoc et al. 2006; Mikkelsen et al. 2007). H3K9me3 is usually associated with H3K20me3 at heterochromatic locations such as pericentromeric chromatin, but this bivalent mark is absent at subtelomeric regions and gene deserts suggesting different silencing mechanisms at these different heterochromatic regions (Rosenfeld et al. 2009). In addition to its heterochromatin formation function, H3K9me2/3 is implicated in the silencing of euchromatic genes. RB and KAP1 corepressor complexes recruit lysine HMTs SUV39H1 and ESET/SETDB1 respectively to promoters of active genes. HP1 is recruited to sites of

H3K9 methylation but is restricted to the promoter region of genes and does not spread (Kouzarides and Berger 2007). The role of H3K9me3 in the coding region of genes has not been elucidated, but enrichment of H3K9me3 at the 3′ ORF increases and co-localizes with the elongating form of RNAPII during active transcription. Moreover, despite the accepted dogma that HP1 is thought to always be repressive, a γ-isoform of HP1 has been found to also be enriched in the coding regions of active genes (Vakoc et al. 2005). During transcriptional activation, promoter repression by HP1β is replaced by HP1γ, which seems to facilitate RNAPII processivity through the coding region of the gene in addition to an increase in H3K9me3 (Mateescu et al. 2008).

13.2.4.6 H3K20

In addition to H3K9me2/3, H4K20me3 is also indicative of silenced chromatin. H4K20 methylation is catalyzed by two SET-domain containing lysine HMTs, SUV4-20H1 and SUV4-20H2. Interestingly, both of these HMTs have been shown to interact with the repressive HP1 isoforms, α and β, indicating a possible upstream function for H3K9 methylation and subsequent H4K20 methylation (Schotta et al. 2004). This idea is further illustrated by the dual enrichment of H3K9me3 and H4K20me3 at constitutively repressed regions such as transposons, satellite and long terminal repeats (LTRs), and pericentromeric chromatin, a region rich with repetitive satellite elements and interspersed with long and short interspersed nuclear elements (LINEs and SINEs). As discussed in the previous section, gene deserts are enriched with H3K9me2/3 but not H4K20me3. Interestingly, neither mark is found at telomeric and subtelomeric regions, which suggests a different mechanism of repression mediates constitutive heterochromatin at telomeres (Rosenfeld et al. 2009).

In contrast to H4K20me3, H4K20me1 is associated with highly expressed genes and is enriched at the 5′ coding region along with H2BK5me1, H3K4me1/2/3, H3K9me1, H3K27me1, and H3K79me1/2/3 (Wang et al. 2008). As previously discussed, H3K36me3 is located at the 3′ end of the coding region and marks transcriptionally active genes. Studies have shown that H4K20me1, H3K36me3, and H3K79me1/2/3 facilitate transcriptional elongation as all three marks fluctuate in a similar temporal manner during gene activation and subsequent transcription (Vakoc et al. 2006). H4K20me2 also seems to be required for checkpoint function and cell survival after DNA damage through the recruitment of Tudor-domain containing protein Crb2 (Greeson et al. 2008).

13.2.5 Histone Demethylases

Reversal of histone methylation was thought to be impossible due to the stable nature of the modification until the discovery of lysine-specific demethylase 1 (LSD1). LSD1 is a FAD dependent amine oxidase that catalyzes lysine demethylation

and releases the product hydrogen peroxide (Shi et al. 2004). Protein arginine deiminase 4 (PADI4) converts methyl-arginine to citrulline rather than an unmodified arginine. PADI4 does not complete full demethylation and therefore requires processing by histone replacement or aminotransferases for complete arginine demethylation (Bannister et al. 2002). Lastly, the JumonjiC-domain containing histone demethylases (JHDMs) are Fe^{2+} and α-ketoglutarate dependent histone demethylases that release the product formaldehyde (Tsukada et al. 2006). Specifics about individual enzymes, mechanisms, specificity, and transcriptional activity can be found in Table 13.2.

13.2.6 Histone Proteolysis

In addition to demethylation and deacetylation, previous reports of H3 N-terminal tail proteolytic cleavage have also been described as a mechanism that facilitates the removal of HPTMs (Allis et al. 1980). Recently, H3 tail cleavage by Cathepsin L has been linked to transcriptional activation and induction of differentiation in embryonic stem cells. N-terminal tail cleavage is also regulated by the HPTMs present on the tail (Duncan et al. 2008). Studies have shown (Santos-Rosa et al. 2009) that cleavage is inhibited by the activation mark H3K4me3 and facilitated by the repressive mark H3R2me2 suggesting that tail clipping is a rapid way to void promoters of repressive marks and complexes during the regulation of gene expression. Moreover, tail clipping directly precedes histone eviction at promoters, which provides strong evidence that H3 tail cleavage is a gene activating event (Santos-Rosa et al. 2009). A major challenge in the chromatin field remains in understanding how patterns of modifications are generated and interpreted by nuclear machinery.

13.2.7 Histone Crosstalk

Given all the histone modifications discussed in the previous sections, regulation of chromatin structure and transcriptional activity can be tightly controlled through the use of combinatorial modifications (Zhang and Reinberg 2001). Histone modifications can affect the stimulation or inhibition of multiple cellular processes, which subsequently affects the capacity for the creation or erasure of other HPTMs (Fig. 13.3) (adapted from Bannister and Kouzarides 2011). Some modifications can inhibit the targeting of other modifications as seen with H3K27, which can be exclusively methylated or acetylated. Various modifications are also dependent on one another. For example, H2B120 ubiquitylation is necessary for H3K79 methylation in both yeast and humans (Lee et al. 2007a; Kim et al. 2009b). Modifications can also prevent the binding of certain effector proteins as is the case with the inhibition of HP1′s targeting to H3K9me2/3 by H3S10 phosphorylation (Fischle et al. 2005). Some marks can also facilitate the binding of effector proteins that in turn perform

Table 13.2 Histone demethylases

Enzymatic family	Subfamily	Enzymes	Specific residue activity	Transcriptional activity	References
PADI		PAD4	H3R2me1 H3R8me1 H3R17me1 H3R26me1 H4R3me1	Derepressors	Bannister et al. (2002), Wang et al. (2004b), and Cuthbert et al. (2004)
Amine oxidase	LSD1		H3K4me1/2 H3K9me1/2	Repressors: CoREST, NuRD Activator: AR/ERα	Lee et al. (2005), Shi et al. (2005), Wang et al. (2009), Metzger et al. (2005), and Garcia-Bassets et al. (2007)
JmjC	JHDM1	JHDM1A JHDM1B	H3K36me1/2		Tsukada et al. (2006)
	JHDM3/JMJD2	JMJD2/JHDM3A JMJD2B JMJD2C/GASC1 JMJD2D	H3K9me2/3 H3K36me2/3		Whetstine et al. (2006), Klose et al. (2006), Cloos et al. (2006), and Fodor et al. (2006)
	JARID	JARID1A JARID1B JARID1C JARID1D	H3K4me2/3	Repressor of growth inhibitors	Iwase et al. (2007), Klose et al. (2007), Lee et al. (2007b), and Yamane et al. (2007)
	UTX/UTY	JMJD3 UTX	H3K27me2/3	Activator: MLL	Agger et al. (2007) and Issaeva et al. (2007)
	JHDM2	JHDM2A JHDM2B JHDM2C	H3K9me1/2	Activator: AR	Yamane et al. (2006)

Acronyms: *PADI* Peptidyl arginine deiminase, *LSD* Lysine specific demethylase, *JmjC* Jumonji C, *JHDM* JmjC-domain-containing histone demethylase, *AR* Androgen receptor, *ER* Estrogen receptor, *CoREST* Corepressor for RE1 silencing transcription factor/neural-restrictive silencing factor, *NuRD* Nucleosome remodeling and histone deacetylase

other modifications. As mentioned above, ING2 contains a PHD finger domain that binds H3K4me3 (Champagne and Kutateladze 2009) in response to DNA damage. Once Sin3a is recruited, HDAC1 activity is stimulated to deacetylate histones and reduce transcriptional activity of genes that promote cell growth and division (Shi et al. 2006).

13.3 Epigenetics and Human Disease

The human body is comprised of trillions of cells, each of which concurrently performs a specific function in order to form a functional human being. The function that one cell serves may be drastically different from another, yet each cell contains identical genetic information. Such phenotypic diversity is a result of a cell's distinctive gene expression profile. Gene expression is directly influenced by various factors including histone modifications, DNA methylation, histone variants, and availability of functional chromatin modifying complexes. Occasionally, DNA sequences targeted for modifications are expanded or contracted, or the enzymes that catalyze the addition or removal of modifications are lost or mutated. Respectively, these events cause a redistribution of DNA methylation and histone modification patterns. Alteration in the localization of these marks at sites such as promoters, repeat elements, and constitutive heterochromatin ultimately result in diseased states due to dysregulated gene expression (Kaufman and Rando 2010).

The idea that influences beyond the genetic code could determine phenotype is not by any means novel. In 1942, C.H. Waddington coined the phrase "epigenetic landscape" to denote changes in phenotype during development despite an identical genotype (Waddington 1957). To date, the epigenetic landscape portrayed by Waddington could be described by two important areas of chromatin research: the elaborate patterns of histone modifications and histone variant substitutions coined, "the histone code" (Jenuwein and Allis 2001) and DNA methylation patterns (Bird and Wolffe 1999). Through its direct effects on transcriptional regulation, histone modifications and DNA methylation affect many essential cellular processes such as embryogenesis, genomic imprinting, DNA replication, microRNA expression, and X-chromosomal inactivation.

Evidence that some human diseases are caused by something other than just the genes you possess is seen in cancer (Esteller 2007), autoimmune disorders (Javierre et al. 2010), and health related issues such as type 2 diabetes (Miao et al. 2008), coronary artery disease (Ordovas and Smith 2010), and obesity (Campion et al. 2009), to name a few. The role of epigenetics in the development of disease is further illustrated by the discordance of disease and trait development in monozyotic twins. Based on this study, environmental factors seem to play a significant role in disease susceptibility and dictating an individual's epigenetic landscape (Fraga et al. 2005). Ultimately, an increase in disease susceptibility can be attributed to environmentally influenced differences in DNA methylation and histone modification patterns that affect levels of gene expression.

With so many new advents in biomedical research, using human epigenetic profiling for understanding disease and even developing medical treatments has never seemed so tangible. Genome-wide association studies (GWAS) and high-throughput sequencing has allowed for high resolution comparison of modifications and gene expression in various organisms. With a future understanding of the basic functional roles these modifications play as transcriptional regulators in the cell, development of targeted treatments resulting in artificial epigenetic landscaping can potentially be established.

13.4 Summary

Despite the rapid progression of discoveries in the epigenetics fields, there still remain many obstacles and questions left unanswered. Several HPTMs have been discovered without finding the enzyme or complex that performs the covalent addition onto histones or its' removal. Some chromatin modifications are scarce enough that studying them would be impossible without new nanotechnologies such as ChIP-seq, RNA-seq, and MeDIP-seq. However, a problem that many GWAS run into is that many modifications are context dependent. Frequently, experiments performed to locate modifications and their effects on transcription produce results that represent a static state for a specific cell type. Both the cell type and time point at which the data was collected also affects what genes are expressed. Moreover, as in the case of H3K9 methylation, some modifications have the ability to alter a gene's 3D spatial positioning within the nucleus (Guelen et al. 2008). Therefore, in addition to the direct effects that chromatin modifying complexes and covalent histone modifications have on promoters, another layer of complexity is added to transcriptional regulation by the way of a gene's spatiotemporal positioning and organization within the nuclear architecture.

In this chapter, several classes of chromatin modifications and their subsequent effects on transcription have been described. There are many other mechanisms of transcriptional regulation that were mentioned but not discussed including arginine methylation, ubiquitylation, deiminination, and sumoylation. Although the enzymes that catalyze many of these and previously discussed reactions have been discovered, the mechanisms by which they control transcription, are established during development, and are stably maintained in somatic cells are still unclear. Albeit, many modifications have been characterized by their individual effects on gene transcription, developing a more complete picture of the complex orchestration between the enzymes that catalyze the reactions of chromatin modifications will lead to a better understanding of transcriptional regulation. Elucidating the code behind the interplay between chromatin modifying complexes and HPTMs provides exciting new prospects for development of medical treatments in the future that will target chromatin modifying enzymes.

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Chapter 14
Histone Variants and Transcription Regulation

Cindy Law and Peter Cheung

Abstract Histones are the protein components of chromatin and are important for its organization and compaction. Although core histones are exclusively expressed during S phase of the cell cycle, there exist variants of canonical histones that are expressed throughout the cell cycle. These histone variants are often deposited at defined regions of the genome and they play important roles in a variety of cellular processes, such as transcription regulation, heterochromatin formation and DNA repair. In this chapter, we will focus on several histone variants that have been linked to transcription regulation, and highlight their physical and functional features that facilitate their activities in this context.

14.1 Overview of the Biology of Histones

14.1.1 Formation of Chromatin

DNA is the genetic material for all living organisms. When stretched out, the DNA in a human cell is approximately 2 m long. Therefore, a sophisticated mechanism is required to organize the DNA so that it can be stored in a nucleus that is only a few microns in diameter, but also still be accessible for biological functions. In eukaryotic cells, chromatin is formed through the physical association of DNA and histone proteins, and is organized through multiple levels of compaction. At the lowest compaction level, 147 bp of DNA is wrapped around two copies-each of the four core histones, H2A, H2B, H3 and H4, to form the nucleosome core particle

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(Kornberg 1974). Adjacent nucleosomes are linked together by short stretches of linker DNA to form the "beads on a string" structure, which is also referred to as the 10 nm fibre. With the addition of the linker histone H1, chromatin is further assembled into the 30 nm fibre under physiological conditions. The 30 nm fibre can coil to form solenoid structures, and upon further higher order folding, the most condensed form of chromatin is the metaphase chromosome (Alberts 2002).

14.1.2 Canonical Histones vs. Histone Variants

Histones are amongst the most conserved proteins in all eukaryotes (Pusarla and Bhargava 2005). Core histones (also known as canonical histones) are encoded by multicopy genes that are intronless, and are transcribed into mRNA that are not polyadenylated (Albig and Doenecke 1997). Instead of a poly-A tail, canonical histone transcripts contain a conserved 3′ stemloop that is responsible for their restricted expression at S phase, a time when additional histones are needed to associate with the newly synthesized DNA (Harris et al. 1991; Dominski and Marzluff 1999). Histone variants are non-allelic isoforms of core histones that differ in parts of their amino acid sequences compared to their core counterparts. Unlike core histones, histone variants are encoded by single-copy genes that contain introns. Also, histone variant mRNAs are polyadenylated and are constitutively transcribed at all stages of the cell cycle. This last feature suggests that they have additional functions beyond DNA compaction (Kamakaka 2005). While core histone deposition is replication-dependent, most histone variants are deposited in a replication-independent manner. All core histones have variant counterparts: for example, a variant form of H4 was very recently identified in human adipocytes (Banaszynski et al. 2010; Jufvas et al. 2011). Most H2B variants are also tissue-specific and have specialized functions in the testes of vertebrates and invertebrates (Ausió 2006). Finally, variants of H3 and H2A are more ubiquitously found and are involved in diverse functions in the cell (Ausió 2006).

14.1.3 Histone Variants and PTMs: Formation of Chromatin Domains

Higher-order chromatin folding reduces accessibility of DNA, yet local access of defined DNA sequences by nuclear factors is essential for transcription, replication and DNA repair. Therefore, the mechanisms that efficiently compact chromatin must also allow for dynamic decondensation at functional regions of the genome. Two general ways to regulate DNA accessibility are by exploiting changes in histone-DNA and histone-histone interactions through post-translational modifications (PTMs) on histones, as well as by incorporation of histone variants (Strahl and Allis 2000;

Henikoff and Ahmad 2005). Additionally, changes to chromatin can also be mediated through physical sliding or removal of nucleosomes by the actions of ATP-dependent chromatin remodelling complexes (Lusser and Kadonaga 2003).

Histones are subjected to a variety of PTMs, including acetylation, methylation, phosphorylation and ubiquitylation. These modifications primarily occur on the N-terminal tails of histones that protrude out from the nucleosome, but can also occur at sites within the histone fold domains, as well as the C-terminal tails of H2A and H2B (Kouzarides 2007). Histone modifications are involved in diverse functions such as epigenetic inheritance, gene expression, heterochromatinization, and the formation of specialized regions such as telomeres and centromeres (Talbert and Henikoff 2010). The addition of different PTMs on histones can alter the interactions between nucleosome and DNA, or between nucleosomes (Kouzarides 2007). They can also create binding sites for nuclear factors and regulate recruitment of protein complexes that mediate downstream functions (Taverna et al. 2007).

Histone variants can replace core histones at defined regions of the genome to confer unique functions. Since histone variants differ in parts of their amino acid sequences compared to core histones, they may also have distinct modifications that recruit different proteins to these regions (Taverna et al. 2007). Together, the incorporation of histone variants and histone PTMs can create structurally and functionally distinct chromatin domains. Chromatin is often categorized into two main compaction states: euchromatin and heterochromatin. Euchromatin has an open and decondensed structure, and are enriched for active genes. Consistent with that, histone variants and modifications that are associated with transcriptional activation are often found in the euchromatic regions of the genome (Kouzarides 2007). Conversely, regions of heterochromatin are compact and condensed in structure. These regions contain inactive genes, and are enriched for different sets of histone variants and modifications that establish a transcriptionally repressive state of chromatin (Kouzarides 2007). As variants of H3 and H2A are most often associated with distinct transcriptional states of chromatin, we will focus on these variants in this chapter.

14.2 The Role of H3.3 in Transcription Regulation

14.2.1 Overview of H3 Variants

Histone H3 has two canonical variants, H3.2 and the mammalian-specific H3.1 (Marzluff et al. 2002). The expression and deposition of these core H3 variants are replication-dependent (Osley 1991). Since H3.1 and H3.2 only differ by one amino acid, most studies tend to group these variants together. For this reason, and for simplicity, we will use the general term H3 to refer to both of these variants in this section. In addition of canonical H3, there are four replacement H3 variants: the centromere-specific variant, CENP-A; two testis-specific variants, H3t and H3.5; and the transcription-linked H3.3. CENP-A is the variant that substitutes for H3 at

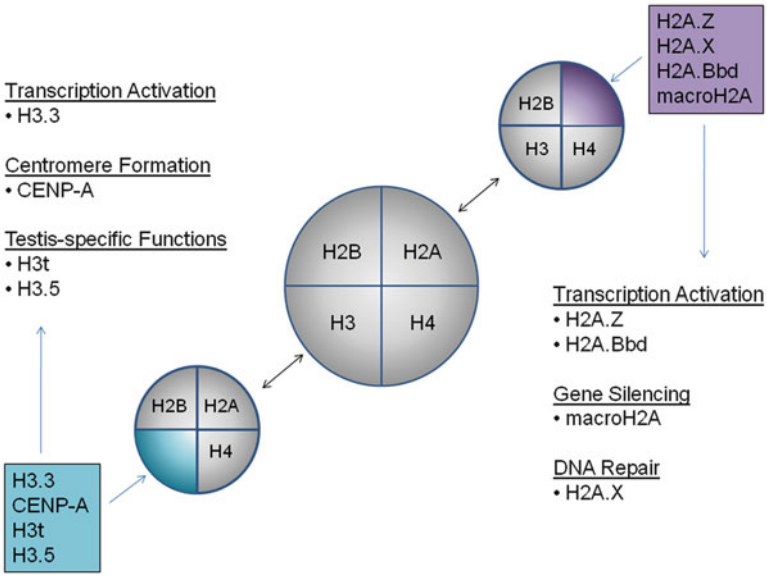

Fig. 14.1 Incorporation of histone variants mediates various cellular processes. When histone variants replace canonical histones in the nucleosome, they may impart distinct functions. The functions of various histone H3 and H2A variants are highlighted in this figure

centromeric nucleosomes. It is required for the recruitment of proteins essential for kinetochore formation and for chromosome segregation (Sullivan et al. 1994). H3t is only expressed in testes and has four additional amino acids compared to H3.1 (Hake and Allis 2006). H3.5 is also a testis-specific variant. It has a ~96% sequence identity with H3.3 and it could rescue H3.3 deficiency in knockdown experiments; however, whether it specifically compensates H3.3's function in transcription is unknown (Schenk et al. 2011). Finally, amongst all the H3 replacement variants, H3.3 is the best studied, and the most clearly linked to transcription regulation (Fig. 14.1).

14.2.2 H3.3 vs. Canonical H3

H3.3 differs from H3.2 at amino acid residues 31, 87, 89 and 90, and it differs from H3.1 at an additional residue at position 96 (Akhmanova et al. 1995; Szenker et al. 2011). This variant is deposited in a replication-independent manner and it is the predominant form of H3 expressed in quiescent, G1 and G2 cells (Frank et al. 2003). The difference in sequence at residues 87–90 between H3 and H3.3 is responsible for this replication-independent deposition. In *Drosophila*, any amino acid substitution of H3 towards the H3.3 residues at these positions allows some H3 deposition in a replication-independent manner (Ahmad and Henikoff 2002).

Table 14.1 Chaperones and genomic location of H3.3, H2A.Z, H2A.Bbd and MacroH2A

Histone variant	Chaperone	Genomic location	Variant/ Chaperone knockout phenotype	References
H3.3	HIRA	Euchromatin, Promoter and gene bodies	HIRA: Embryonic lethality	Roberts et al. (2002)
H3.3	Daxx-ATRX	Telomeres, Pericentric heterochromatin	Daxx-ATRX: Embryonic lethality	Michaelson et al. (1999) Garrick et al. (2006)
H2A.Z	Chz1, Swr1	Promoter, Boundary elements, Pericentric heterochromatin	H2A.Z: Embryonic lethality Swr1: Lethality	Faast et al. (2001) Updike and Mango (2006)
H2A.Bbd	Unknown	Unknown	Unknown	–
MacroH2A	Unknown	Inactive X chromosome, Promoter	MacroH2A: Malformations in the brain and body (zebrafish)	Buschbeck et al. (2009)

This region may also be important for specific chaperone recognition since H3 and H3.3 are deposited into chromatin by different chaperones. HIRA, a nucleosome assembly factor, is found to specifically deposit H3.3 at the promoter and coding regions of active genes in a replication-independent manner, whereas H3.1 is exclusively deposited by CAF-1 during DNA replication (Tagami et al. 2004; Goldberg et al. 2010). More recently, another chaperone, Daxx, in a complex with ATRX, was found to deposit H3.3 at telomeres in murine embryonic stem cells and at pericentric heterochromatin in mouse embryonic fibroblasts (Lewis et al. 2010). For now, the function of H3.3 at telomeres is not known, nor is it clear whether there are distinguishing characteristics on the H3.3 pools associated with the different chaperone complexes (Table 14.1).

14.2.3 Evidence Linking H3.3 to Transcriptional Activation

Genome-wide mapping of H3.3 in *Drosophila, C. elegans* and mammalian cells revealed that H3.3 is mostly enriched at actively transcribed genes (Mito et al. 2005; Ooi et al. 2010; Delbarre et al. 2010). While some studies showed that H3.3 is only found at the promoters of genes, others found enrichment of H3.3 at both the promoter and coding region (Chow et al. 2005; Daury et al. 2006). ChIP-chip experiments showed that the peaks of H3.3 localization overlap with the genomic regions enriched for K4-methylated H3 and RNA Pol II, which are both marks of active transcription (Mito et al. 2005). Indeed, biochemical and mass spectrometry analyses showed that this variant is enriched for transcription-associated PTMs, such as

methylation on K4, K36, and K79 and acetylation on K9, K14, K27 and K79 (McKittrick et al. 2004; Hake et al. 2006; Loyola et al. 2006). All evidence together suggest that the majority of H3.3 is physically associated with active genes.

14.2.4 H3.3 and Transcription Regulation

To determine H3.3's function, various labs have examined the dynamics of H3.3 incorporation during transcriptional activation. For example, using inducible gene expression systems, it was found that transcriptional activation is accompanied by displacement of H3 and selective re-deposition of H3.3 at these genes (Schwartz and Ahmad 2005; Wirbelauer et al. 2005; Sutcliffe et al. 2009). In spite of the steady-state distribution of H3.3 at promoters and coding regions, Tamura et al. found that transcription-induced incorporation of H3.3 is greatest at the distal end of the coding region of interferon-stimulated genes upon gene induction, suggesting that H3.3 replaces the evicted canonical H3 during transcription (Tamura et al. 2009). In addition, knockdown of H3.3 or HIRA, the main H3.3 chaperone, inhibited induction of H3.3-target genes, indicating that H3.3 is required for gene activation (Placek et al. 2009; Tamura et al. 2009). Together, these results suggest that H3.3 incorporation is necessary for the rapid exchange of H3 components and possibly for depositing transcription-linked PTM signatures on the H3 at induced genes.

14.2.5 Properties of H3.3 Nucleosomes

Incorporation of H3.3 can alter the biophysical properties of the nucleosome, either by nucleosome destabilization, which would facilitate its rapid removal, or by changing the interactions of the nucleosome with DNA or other nucleosomes, which would antagonize formation of higher-order chromatin structures. Enrichment of H3.3 at transcription start sites (TSS) is inversely correlated with the deposition of histone H1, a linker histone involved in chromatin compaction. Knockdown of H3.3 in *Drosophila* cells by siRNA results in increased H1 binding at TSS, as well as increased nucleosome repeat length, suggesting that H3.3 nucleosomes inhibit further compaction of chromatin (Braunschweig et al. 2009). H3.3-nucleosomes are also more sensitive to salt-dependent disruptions and, compared to H3-nucleosomes, are more prone to losing their H2A/H2B dimers. Such increased nucleosome mobility would allow the H3.3-nucleosomes to be remodelled rapidly during transcriptional activation (Jin and Felsenfeld 2007).

Although much has been learnt about H3.3 over the last decade, there are also interesting questions regarding this variant that remain unresolved. For example, with only four/five amino acids that differ between canonical H3 and H3.3, how do histone chaperones and modifying enzymes differentiate between them? Given that H3.3, H3.2 and H3.1 have distinct PTM signatures, is this due to preferential

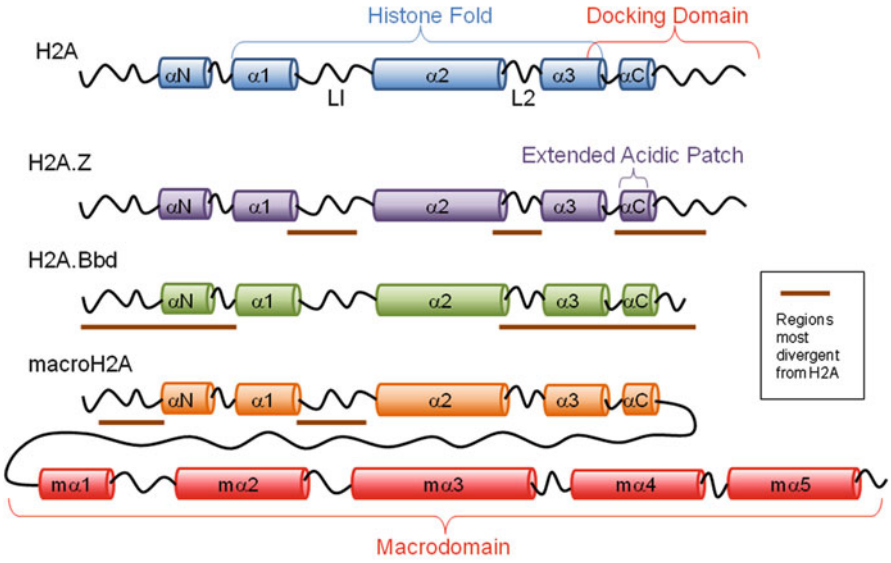

Fig.14.2 Schematic diagram of the secondary structures of histone H2A and H2A variants. The α-helices are represented by *cylinders*. The histone fold, docking domain and the L1 and L2 loops are labelled on the H2A schematic. The *bars* below the H2A variant schematic highlight regions that are most divergent from H2A. H2A.Z has significant differences in the L1 loop region that is important for interactions between the two H2A/H2A.Z-H2B dimers, as well as in the docking domain that is important for interactions between H2A.Z and the H3-H4 tetramer. H2A.Z also contains an extended acidic patch that is important for interactions with other nucleosomes. H2A.Bbd is most divergent from H2A in the N- and C-terminal regions. H2A.Bbd lack regions corresponding to the docking domain and the acidic patch of canonical H2A. MacroH2A differs from H2A in the L1 loop region and also contain a macrodomain that encompass two-third of the size of this histone

recognition by distinct histone modifying enzymes or due to the differential positioning of the variants at different regions of the genome (Hake et al. 2005)? Finally, new evidence shows that H3.3 is also found at intergenic regions, telomeres and centromeres (Mito et al. 2005; Wong et al. 2009; Drané et al. 2010; Santenard et al. 2010). So what are the functions of H3.3 at these regions that are often thought to be transcriptionally silent? These remaining questions will spur on the current and future research on H3.3.

14.3 Variants of Histone H2A

The Histone H2A family of variants consists of H2A.X, H2A.Z, macroH2A and H2A.Bbd. Whereas H2A.Z is conserved throughout evolution, the other variants, such as macroH2A and H2A.Bbd, are only found in vertebrates or mammals (Ausió 2006). H2A.X has a critical role in the process of DNA repair. Phosphorylation of H2A.X

marks the site of DNA damage on chromatin and physically recruits DNA repair factors to the DNA breaks (Rogakou et al. 1998). Since the function of H2A.X in DNA repair is well documented, we will refer interested readers to the many excellent reviews already published (Foster and Downs 2005; Thiriet and Hayes 2005).

14.3.1 H2A.Z – The Essential H2A Variant

H2A.Z is one of the best-studied H2A variants and represents about 5–10% of total cellular H2A. H2A.Z is highly conserved throughout evolution with a sequence identity of ~90% between species, whereas its sequence identity to canonical H2A is only about ~60% (Iouzalen et al. 1996; Jackson and Gorovsky 2000) (Fig. 14.2). H2A.Z has an essential function in complex eukaryotes since mutants that are genetically null for H2A.Z in *Tetrahymena*, *Drosophila* and mice are all inviable (van Daal and Elgin 1992; Gorovsky 1996; Faast et al. 2001). H2A.Z has been implicated in a variety of biological processes, including transcriptional activation, heterochromatin formation, chromosome segregation and regulation of cell cycle progression (Zlatanova and Thakar 2008). At present, which of these putative functions of H2A.Z is essential to cell survival is still unclear.

14.3.1.1 Evidence Linking H2A.Z to Transcriptional Activation

H2A.Z has long been thought to have a transcriptional activation function since initial observations showed that it is only found in the transcriptionally active macronucleus of *Tetrahymena* (Allis et al. 1980). In yeast, genome-wide analyses from different groups showed that H2A.Z is enriched at promoters and often flank nucleosome-free regions adjacent to the transcription start site of many genes (Zhang et al. 2005a; Guillemette et al. 2005; Li et al. 2005; Millar et al. 2006). Correlation of the genome-wide localization studies with gene expression data showed that H2A.Z in *S. cerevisiae* is mostly enriched at inactive or repressed genes. However, loss of H2A.Z does not de-repress these genes, but disrupt their inducibility. Therefore, H2A.Z in yeast, does not have a direct transcriptional repression function, but may be required to poise inducible genes for activation (Santisteban et al. 2000; Adam et al. 2001). In contrast to the yeast studies, ChIP-seq analyses by the Zhao lab using human T cells showed that there is an enrichment of H2A.Z at the promoters of actively transcribing genes (Barski et al. 2007). The contrasting observations in yeast and human cells have not been reconciled at present.

Early studies in yeast showed that when genes are activated, their promoters are remodelled to lose H2A.Z. Htz1 (the H2A.Z gene in *S. cerevisiae*) deletion mutants are viable but defective in the activation of H2A.Z-target genes and they exhibit a slow growth phenotype (Santisteban et al. 2000; Adam et al. 2001). In mammalian cells, a number of studies showed that H2A.Z is already present at the regulatory

regions of some inducible genes (Farris et al. 2005; Gévry et al. 2007; Sutcliffe et al. 2009; Draker et al. 2011), but gene activation-dependent recruitment of H2A.Z has also been reported (Hardy et al. 2009; Gévry et al. 2009). Similar to yeast studies, a net loss of H2A.Z is often observed after transcriptional induction of mammalian genes (Draker et al. 2011; Hardy et al. 2009; Gévry et al. 2009; Amat and Gudas 2011). Finally, shRNA-mediated knockdown of H2A.Z in different cell lines showed that this variant is required for gene activation (Draker et al. 2011; Gévry et al. 2009; Cuadrado et al. 2010). At present, a consensus model of how deposition and eviction of H2A.Z is involved in transcription regulation has remained elusive; however, the loss of function studies clearly showed that H2A.Z is required for the activation of many genes in both yeast and mammalian cells. In support of this, deletion or knockdown of SRCAP (SWR1 in yeast), an ATPase responsible for H2A.Z deposition, causes decreased H2A.Z deposition as well as transcriptional defects, confirming that H2A.Z has an critical role in transcription regulation (Santisteban et al. 2000; Kobor et al. 2004; Wong et al. 2007; Slupianek et al. 2010).

14.3.1.2 Mechanism of H2A.Z Function: Poising Promoters for Transcription

Structural analyses of the H2A.Z nucleosome showed that it differs from the canonical nucleosome at the H3/H4 docking domain, and this change is thought to destabilize the H2A.Z/H3 interaction (Suto et al. 2000). Sedimentation analyses under changing ionic strength showed a substantial instability of the H2A.Z core particle, indicating a less tight binding of the H2A.Z-H2B dimer to the rest of the octamer (Abbott et al. 2001). Consistent with these stability assays, additional studies showed that incorporation of H2A.Z increases the mobility of the nucleosome. For example, studies in yeast showed that H2A.Z nucleosomes have higher turnover rates (Dion et al. 2007). Also, *in vitro* thermal mobility assays showed that H2A.Z nucleosomes are more mobile at increasing temperatures as compared to H2A nucleosomes, suggesting that H2A.Z nucleosomes may be more easily remodelled (Flaus et al. 2004). Since H2A.Z is preferentially found at the promoters of repressed genes and is lost upon gene activation, the increased mobility of H2A.Z nucleosomes may be important for maintaining genes in a poised state and allow rapid nucleosome remodelling upon induction.

Biophysical studies of chromatin fibres containing H2A.Z showed that it resists condensation and assumes a more relaxed conformation as compared to ones containing the H2A counterpart (Fan et al. 2002). Sedimentation velocity experiments showed that H2A.Z facilitates intramolecular folding of nucleosomal arrays, as in the formation of 30 nm fibre (Fan et al. 2002). The extended acidic patch on H2A.Z is important for its interactions with the N-terminal tail of H4 from a neighbouring nucleosome, which may allow H2A.Z to facilitate the folding of the DNA (Suto et al. 2000). At higher salt concentrations, H2A.Z nucleosomal arrays sedimented more slowly when compared to control arrays. This indicates that H2A.Z inhibits the oligomerization of the array, and suggests that H2A.Z inhibits the formation

of highly condensed structures that would result from intermolecular interactions (Fan et al. 2002). The incorporation of H2A.Z is thought to resist permanent silencing of genes by inhibiting heterochromatin formation and, thus, keep genes poised for transcription (Meneghini et al. 2003). Indeed, deletion studies in yeast showed that loss of H2A.Z results in spreading of the telomeric heterochromatin into the adjacent euchromatic regions, suggesting that it has an anti-silencing function. In addition, H2A.Z also functionally antagonizes DNA methylation in *Arabidopsis*, so it could protect promoter regions from being silenced by DNA methylation (Zilberman et al. 2008). In line with H2A.Z's function in keeping promoters poised, H2A.Z can also affect nucleosome positioning at the promoters of target genes (Guillemette et al. 2005). Nucleosome mapping of the TFF1 promoter showed that knock-down of H2A.Z impairs stabilization of nucleosomes at the promoter. The adoption of preferential sites by nucleosomes at the TFF1 promoter allows ERα and its partners to interact with their binding sites (Gévry et al. 2009). Therefore, incorporation of H2A.Z may mediate nucleosome positioning that sets up a chromatin environment that favours binding of transcription-associated proteins.

14.3.1.3 Mechanism of H2A.Z Function: Recruitment of Transcription Machinery

H2A.Z's role in transcription regulation is not only limited to setting up the chromatin environment and for rapid nucleosome exchange, but it may also be important for direct recruitment of components of the transcription machinery. In yeast, loss of H2A.Z results in defective recruitment of RNA Pol II and TBP to the GAL1 promoter. Physical interaction between RNA Pol II and the C-terminal tail of H2A.Z, shown through biochemical analyses, further suggests a direct recruitment function of H2A.Z in transcriptional initiation (Adam et al. 2001). Recent studies by Santisteban et al. showed that H2A.Z is also important for transcriptional elongation. They found that nucleosome remodelling over the coding region during transcription requires H2A.Z. In the absence of H2A.Z, RNA Pol II elongation rate is 24% slower and RNA Pol II phos-Ser2 levels are reduced as compared to WT strains (Santisteban et al. 2011). H2A.Z has also been linked to the recruitment of RNA Pol II to the promoter in mammalian cells since knock-down of H2A.Z impaired RNA Pol II recruitment to the IL8 promoter upon gene induction (Hardy et al. 2009). Therefore, H2A.Z may regulate gene expression at multiple steps, including promoter poising, transcriptional initiation and elongation.

14.3.1.4 Relationship Between H3.3 and H2A.Z

Several recent studies have suggested an interesting relationship between H2A.Z and H3.3. H3.3 and H2A.Z are both found at promoters and are both involved in nucleosome positioning at these regions (Jin et al. 2009; Thakar et al. 2009). When

studied individually, both variants are involved in transcriptional activation and their incorporation destabilizes the nucleosome. Genome-wide studies showed that both H3.3 and H2A.Z are enriched at enhancers and insulator regions (Mito et al. 2005; Barski et al. 2007). Moreover, salt extraction studies showed that nucleosomes containing both H3.3 and H2A.Z are less stable than nucleosomes with just H3.3 and H2A (Jin and Felsenfeld 2007). Therefore, the two variants may cooperate and synergize at specific promoters to allow rapid remodelling and activation.

14.3.1.5 Heterochromatin Formation and PTMs of H2A.Z

Although most evidence supports H2A.Z's role in transcriptional activation, there are also studies that linked H2A.Z to heterochromatin formation and gene silencing. For example, H2A.Z at pericentric heterochromatin colocalizes with heterochromatin protein 1 α (HP1α) during early mouse development, and *in vitro* studies showed that it promotes the folding of chromatin fiber through an interaction with HP1α (Rangasamy et al. 2003; Fan et al. 2004). Genome-wide analyses also found an enrichment of H2A.Z at facultative heterochromatin (Creyghton et al. 2008; Hardy et al. 2009). The contrasting findings showing H2A.Z's association with both euchromatin and heterochromatin have not yet been clearly resolved. However, approximately 25% of total H2A.Z in mammalian cells is mono-ubiquitylated at K120 or K121 (Sarcinella et al. 2007). Moreover, the mono-ubiquitylated form of H2A.Z is enriched on the epigenetically-silenced inactive X chromosome in human female cells. Therefore, PTMs on H2A.Z may distinguish its association with the different chromatin states.

In addition to mono-ubiquitylation, human H2A.Z is also known to be acetylated on Lys 4, 7 and 11 (Beck et al. 2006). Genome-wide analyses in yeast demonstrated that, whereas H2A.Z is enriched at repressed genes, AcH2A.Z is found at the promoters of active genes (Millar et al. 2006). ChIP analyses in chicken cells also found that AcH2A.Z is associated with active genes (Bruce et al. 2005). Insofar as acetylation of H2A.Z is associated with active transcription, whereas ubiquitylation of H2A.Z is associated with gene silencing, differential modifications by these PTMs may specify the contrasting downstream functions associated with H2A.Z (Draker and Cheung 2009).

14.3.1.6 H2A.Z's Roles in Hormone-Responsive Gene Activation and Links to Cancer

Given H2A.Z's links to transcription regulation, several studies have focused on studying its function in the activation of hormone-responsive genes. Depletion of H2A.Z or of H2A.Z incorporation impedes both estrogen and androgen signalling, and blocks efficient activation of ER- and AR-regulated genes (Gévry et al. 2009; Draker et al. 2011). Moreover, de-ubiquitylation of H2A and H2A.Z correlates with hormone activation of AR-regulated genes, supporting the idea

that modulating the PTMs on H2A.Z could function as a regulatory step in the expression of inducible genes (Draker et al. 2011). Deregulation of ER and AR-activated genes is often linked to the development of breast and prostate cancer respectively. Consistent with the fact that H2A.Z plays important roles in controlling expression of these genes, dysregulation of H2A.Z has also been linked to tumourigenesis. For example, recent studies show that H2A.Z is overexpressed in different cancers and its overexpression correlates with metastatic and undifferentiated cancer types (Zucchi et al. 2004; Hua et al. 2008; Svotelis et al. 2010). These types of cancer are more aggressive and are associated with poorer patient outcome (Rhodes et al. 2004). Therefore, further studies investigating the detailed functions of H2A.Z in gene expression regulation and further identification of H2A.Z-regulated genes will potentially shed light on its role in tumourigenesis.

14.3.2 H2A.Bbd: Variant of Active Transcription

H2A.Bbd is the smallest H2A variant and the most divergent compared to the canonical H2A (only 48% sequence identity) (Fig. 14.2). Its name, Barr body deficient (Bbd), originated from the initial observation that it is excluded from the inactive X chromosome or the Barr body in female mammalian cells. As part of the dosage compensation phenomenon, one of the two X chromosomes in each cell of female mammals is randomly silenced (inactivated) by chromatin compaction and epigenetic mechanisms (see Ng et al. 2007 for more thorough review). Therefore, the conspicuous absence of H2A.Bbd from the silenced inactive X chromosome suggests that it is physically incompatible with silenced or compacted chromatin. Moreover, its preferential colocalization with acetylated H4, a mark of active transcription, further suggests that it has a positive role in transcription regulation (Chadwick and Willard 2001a).

14.3.2.1 H2A.Bbd Lacks the Acidic Patch and the C-terminal Docking Domain of H2A

Structural comparison between H2A and H2A.Bbd showed that the acidic patch of H2A, which is extended in H2A.Z, is absent from H2A.Bbd. Since the acidic patch on H2A is important for the folding of chromatin into the 30 nm fibre, the lack of this region on H2A.Bbd suggests that it may have weaker intermolecular interactions and adopt a more relaxed chromatin structure (Zhou et al. 2007). H2A.Bbd nucleosomal arrays also lack H1 linker histones that are necessary for the formation of higher-order chromatin structures, further suggesting that H2A.Bbd physically antagonizes chromatin compaction, which could serve to facilitate transcriptional activation (Shukla et al. 2010).

Beside the acidic patch, H2A.Bbd also lacks the region corresponding to the H2A C-terminus and part of the docking domain responsible for the interactions between the H2A/H2B dimer and the H3/H4 tetramer (Chadwick and Willard 2001a). H2A.Bbd is less tightly bound to chromatin and the difference in the docking domain is largely responsible for this altered nucleosomal association (Bao et al. 2004; Doyen et al. 2006b). H2A.Bbd nucleosomes are exchanged more rapidly than canonical H2A nucleosomes *in vivo* (Gautier et al. 2004). Recombinant H2A.Bbd histones do not form stable octamers in the presence of the other core histones *in vitro*, but can form nucleosome particles in the presence of DNA (Bao et al. 2004). These biophysical properties alter the stability of H2A.Bbd-containing nucleosomes, and change the amount of DNA wrapped around each nucleosome from 147 bp to ~130 bp (Doyen et al. 2006b). Restriction enzyme analyses also showed increased accessibility in H2A.Bbd nucleosome-containing arrays (Shukla et al. 2010). Therefore, the overall destabilization of the H2A.Bbd nucleosome and nucleosomal arrays is thought to render the chromatin more permissive to active transcription (Zhou et al. 2007). Finally, it is interesting to note that H2A.Bbd only contains 1 lysine compared to the 14 lysine residues in H2A. This unique amino acid composition not only results in a less basic protein, but it also means that H2A.Bbd lacks most of the residues subjected to PTMs on H2A (González-Romero et al. 2008). The significance of this physical feature on the function of this variant remains to be determined.

14.3.2.2 H2A.Bbd: Positive Regulator of Transcription

As a recently discovered variant of H2A, still very little is known about H2A.Bbd. In addition to the previously mentioned studies that link this variant to more accessible chromatin, Angelov et al. found that H2A.Bbd nucleosomes are better substrates for p300-mediated acetylation of histone tails *in vitro*. This suggests that incorporation of H2A.Bbd can enhance acetylation of other histones in the nucleosome context, and further support the prevailing model that H2A.Bbd functions as a positive regulator of transcription (Angelov et al. 2004). As more details of this variant are uncovered in the future, it would be interesting to determine whether H2A.Bbd, like some of the other H2A variants, is also involved in human diseases.

14.3.3 MacroH2A: A Repressive Histone Variant

MacroH2A is a histone H2A variant best known to be associated with gene repression. Directly opposite to H2A.Bbd, immunofluorescence studies showed that macroH2A is enriched at the inactive X chromosome in female mammalian cells (Costanzi and Pehrson 1998). To date, three macroH2A variants have been identified

in mammals. MacroH2A1 and macroH2A2 are encoded by two separate genes and the macroH2A1 transcript is alternatively spliced to give rise to two splice variants: macroH2A1.1 and macroH2A1.2. Although there are subtle differences in the localization of the three variants at autosomes, all three variants are enriched on the inactive X chromosome, indicating they have similar functions (Chadwick and Willard 2001b; Costanzi and Pehrson 2001). Most studies have focused on the more abundant macroH2A1, and unless otherwise indicated, the studies presented below mostly refer to this macroH2A variant.

MacroH2A evolved comparatively recently and is significantly divergent from the canonical H2A. It has an N-terminal histone domain that is only 64% in sequence identity with H2A and it also has an extended 25 kDa non-histone region at the C-terminus (Fig. 14.2). The non-histone region, known as the macrodomain, comprises two thirds of the protein's molecular mass (Pehrson and Fried 1992). The macrodomain of macroH2A has been shown to bind and inhibit the enzymatic activity of poly-ADP-ribose-polymerase 1 (PARP-1) (Ouararhni et al. 2006). Catalytically inactive PARP-1 is involved in the maintenance of heterochromatic regions in *Drosophila*. Therefore, macroH2A and PARP-1 together may be involved in the maintenance of the inactive X chromosome and the silencing of some autosomal genes (Tulin et al. 2002; Nusinow et al. 2007).

14.3.3.1 MacroH2A Is Involved in Gene Silencing

Various localization studies showed that macroH2A is associated with transcriptionally silenced regions of the genome. First, it is enriched on the inactive X chromosome and on the inactive alleles of imprinted genes, all of which are associated with facultative heterochromatin (Costanzi and Pehrson 1998; Choo et al. 2007). Second, macroH2A occupancy at genes negatively correlates with gene expression (Buschbeck et al. 2009; Gamble et al. 2010; Changolkar et al. 2010). Third, genome-wide analyses showed that macroH2A is localized to transcriptionally silenced regions marked by H3K27me3 and PRC2-binding (Buschbeck et al. 2009; Araya et al. 2010). Finally, macroH2A accumulates at senescence-associated heterochromatin foci, which are domains of repressed chromatin associated with cellular aging (Zhang et al. 2005b). All together, these findings show that macroH2A is almost exclusively found at heterochromatic regions of the genome.

In addition to its function in the silencing of the inactive X chromosome, macroH2A also has a role in the silencing of autosomal genes. Not only is macroH2A found at the promoter of the IL-8 gene when this gene is transcriptionally silenced, but knockdown of macroH2A derepressed IL-8 expression, demonstrating that macroH2A is required for gene silencing (Agelopoulos and Thanos 2006). Mechanistically, *in vitro* transcription assays demonstrated that macroH2A represses p300- and Gal4-VP16-dependent transcription. Similar repression is seen when the assay was performed using recombinant H2A histones fused to the macrodomain of macroH2A, suggesting that this defined domain is sufficient, and likely responsible, for the repressive function of macroH2A (Doyen et al. 2006a).

14.3.3.2 Mechanism of MacroH2A-Mediated Repression

Incorporation of macroH2A into the nucleosome confers a unique conformation as indicated by a lower sedimentation coefficient compared to nucleosomes containing core H2A (Changolkar and Pehrson 2002). MacroH2A differs from the canonical H2A in the L1 loop, the region of H2A responsible for the interaction between the two H2A/H2B dimers within a nucleosome (Chakravarthy et al. 2005). Therefore, the distinct L1 loop on macroH2A may alter the stability of the nucleosome. Consistent with that, macroH2A nucleosomes are more tightly bound to chromatin and the core DNA of those nucleosomes are more resistant to DNase I digestion. Such findings suggest that macroH2A is associated with closed chromatin conformation, which is consistent with the repressive role for this variant in transcription regulation (Changolkar and Pehrson 2002; Abbott et al. 2004).

Additional mechanistic studies suggested that macroH2A inhibits binding of transcription factors or chromatin remodelling complexes through steric hindrance of the macrodomain, or by reducing the accessibility of the DNA through alterations of the DNA-nucleosome contacts. The presence of macroH2A in a positioned nucleosome interferes with the binding of the transcription factor NF-kB (Angelov et al. 2003). Studies from Narlikar and colleagues showed that macroH2A is permissive to remodelling by ACF, an ISWI complex implicated in gene repression, but is refractory to chromatin remodelling by SWI/SNF, which is involved in gene activation (Chang et al. 2008). The unique structure of the macroH2A nucleosome may allow direct recognition by chromatin remodelling complexes that are involved in gene repression. Finally, macroH2A binds to HDAC1 and 2 and, thus, could promote transcriptional repression through de-acetylation of the other histones associated with macroH2A (Chakravarthy et al. 2005; Doyen et al. 2006a).

ChIP-chip analyses using a testicular cancer cell line showed that macroH2A colocalizes with repressive marks at the promoters of key developmental genes. *In vivo* studies showed that knockdown of macroH2A in zebrafish embryos results in obvious developmental defects of body formation (Buschbeck et al. 2009). On the other hand, ChIP-chip experiments in primary human fibroblasts have shown that a subset of autosomal genes that are marked by macroH2A are transcriptionally active (Gamble et al. 2010). Therefore, this variant may also have a non-repressive transcriptional function in mammalian cells that remains to be clarified.

In addition to its role in X chromosome inactivation, macroH2A is also important for regulating many other genes. Recent studies further suggested that macroH2A is involved in cancer. Bernstein and colleagues discovered that the loss of macroH2A correlates with a more malignant phenotype in melanoma cells (Kapoor et al. 2010). Mechanistically, macroH2A suppresses tumour progression of malignant melanoma through down regulation of Cdk8. Finally, other studies have found that perturbed ratios of macroH2A variants can be a predictor of lung cancer recurrence (Sporn et al. 2009). All together, these studies provide direct and indirect evidence linking macroH2A to oncogenesis. Not only can macroH2A levels be potentially used as prognostic marker in cancer progression, but it could potentially be directly targeted for therapy.

14.4 Conclusions

Many studies over the last decade have revealed that histone variants function as key regulators of a variety of cellular processes. The mechanisms associated with these variants are only beginning to be elucidated and new discoveries will certainly add to the complexities of their functions. Even new variants are still being discovered very recently, such as the new H4 variant found in human adipocytes, and the additional H2A.Z isoform, H2A.Z-2, which differ from the original H2A.Z by three amino acids (Coon et al. 2005; Jufvas et al. 2011). In addition, some of the well studied variants may also have surprising functions that are less well understood. For example, although CENP-A is better known for its role in kinetochore formation, it may have additional roles in defining a special chromatin environment at the centromeres that is distinct from euchromatin and heterochromatin. Most of the centromere is thought to be transcriptionally silent, but studies by the Jiang group have shown that interspersed between the silent domains lies active genes in rice centromeres (Nagaki et al. 2004). They also found that in addition to the CENP-A variant, the rice centromere also contains active marks of transcription such as H3K4me2 and H4 acetylation (Yan et al. 2006). Similarly, the Earnshaw group showed that H3K4me2 and transcription may be required for proper CENP-A deposition in humans (Bergmann et al. 2011). At present, whether CENP-A has a direct role in transcription is unclear; however, the overall PTM-signatures on CENP-A and associated histones in the nucleosome context likely dictate its specific functions.

While most studies focus on individual histone variants, it is important to recognize that these variants function in the context of nucleosomes. For example, studies by the Felsenfeld group showed that nucleosomes containing both H2A.Z and H3.3 are prone to displacement and may be important for the formation of nucleosome free regions at active promoters (Jin et al. 2009). Analogously, other distinct combinations of histone variants may also exist for specific functions. For example, given that H2A.Bbd and H3.3 are both associated with active transcription, it would be interesting to determine whether distinct types of genes are associated with nucleosomes that have this specific pairing. Similarly, it would be intriguing to test whether histone variants of disparate functions, such as macroH2A and H3.3, can co-exist in the nucleosome context and yield new functional features. Finally, the complexities of these combinations are even more complicated when one takes into account that each nucleosome has two copies each of the four histone types. For example, it is now apparent that H2A.Z not only can pair up with another H2A.Z to form homotypic nucleosomes, but it can also pair with the canonical H2A to form heterotypic nucleosomes (Fig. 14.3). Although the pathways leading to the formation of these homotypic and heterotypic H2A.Z-containing nucleosomes have been delineated in yeast studies (Luk et al. 2010), the functional differences between such nucleosomes are not yet known. Studies by the Henikoff lab have shown that homotypic H2A.Z nucleosomes are enriched downstream of active promoters in *Drosophila*, while heterotypic H2A.Z nucleosomes are depleted at that region

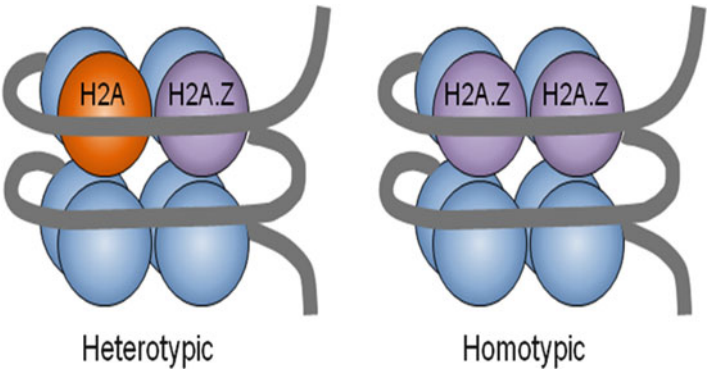

Fig. 14.3 Homotypic and heterotypic nucleosomes. Nucleosomes containing histone variants can exist as homotypic or heterotypic. A homotypic nucleosome contains two copies of the variant histone, in this case two copies of histone variant H2A.Z. A heterotypic nucleosome contains one variant histone, in this case H2A.Z paired with canonical H2A

(Weber et al. 2010), suggesting that they may be functionally distinct. As an extension to the well described "histone code", there may well be a "nucleosome code" or a "histone variant code" that remains to be solved by future studies.

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Chapter 15
Noncoding RNAs in Chromatin Organization and Transcription Regulation: An Epigenetic View

Karthigeyan Dhanasekaran, Sujata Kumari, and Chandrasekhar Kanduri

Abstract The Genome of a eukaryotic cell harbors genetic material in the form of DNA which carries the hereditary information encoded in their bases. Nucleotide bases of DNA are transcribed into complimentary RNA bases which are further translated into protein, performing defined set of functions. The central dogma of life ensures sequential flow of genetic information among these biopolymers. Noncoding RNAs (ncRNAs) serve as exceptions for this principle as they do not code for any protein. Nevertheless, a major portion of the human transcriptome comprises noncoding RNAs. These RNAs vary in size, as well as they vary in the spatio-temporal distribution. These ncRnAs are functional and are shown to be involved in diverse cellular activities. Precise location and expression of ncRNA is essential for the cellular homeostasis. Failures of these events ultimately results in numerous disease conditions including cancer. The present review lists out the various classes of ncRNAs with a special emphasis on their role in chromatin organization and transcription regulation.

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15.1 Overview of ncRNA

Noncoding RNAs (ncRNAs) are a large group of functional RNA transcribed by RNA polymerases, but never translated into protein. A decade before the discovery of these noncoding transcripts, most of the sequences in our genome were believed to be part of "Junk DNA". Soon the genome sequencing projects revealed that only a mere 2% of the human genome codes for protein coding genes and almost 98% of the human transcriptome represents the ncRNA. They include well-characterized transfer RNAs and ribosomal RNAs involved in the process of translation, as well as a huge class of other regulatory ncRNAs which have been shown to play a crucial role in gene regulation. ncRNAs in general function as adaptors for the recognition of a particular nucleotide sequence in the target which is later positioned into the enzymatic molecule associated with the specific class of ncRNA. These functional ncRNA are involved in key cellular processes including transcriptional regulation, RNA processing and modification, protein trafficking, genome stability, mRNA stability, and even protein degradation (Hüttenhofer et al. 2005).

Terminologies like "Junk DNA" and "Transcriptional noise" have been challenged since the discovery of regulatory RNAs that are transcribed by both Pol II and Pol III. The list of known ncRNAs is growing larger in numbers since the completion of whole transcriptome analyses like the ENCODE Pilot Project (Birney et al. 2007), the mouse cDNA project FANTOM, and a series of other large scale transcriptome studies performed to fish these transcribed fragments ('transfrags') using various forms of high throughput tiling arrays, ESTs, SAGE tags and RACE techniques. Ultimately the present scenario of the transcriptome is that ncRNA are vast in number covering a huge proportion of the genome consisting of overlapping, bi-directional transcripts. The major obstacle in the identification of these transcripts is that majority of them are expressed in a short spatio-temporal frame, thus it is difficult to recognize such transcripts even after employing the most advanced deep sequencing techniques. Though the ncRNAs have coexisted with protein coding genes they were kept under curtain mainly because of the model organism taken for such studies. Also the mutations that were considered were often expected to have a major impact on the phenotypic outcome of such genetic screens. Mutation in a protein-coding gene can have severe effects on the structure and function of the protein which ultimately shows an altered phenotype, which is often visible in the regular genetic screening due to the high penetrance. On the other hand recessive mutation phenotypes and single-base mutations are harder to identify in comparison to insertions/deletions (Eddy 2001; Kavanaugh and Dietrich 2009). The reason for a shift in the focus of our research towards protein coding genes is that most of the genetic screening techniques and the methodologies followed during the early era of genomics had an inherent bias toward the scanning of known exons and the flanking sequences in that region. Most of the bioinformatic search tools were based on the signatures of protein coding genic regions which does not hold good for the ncRNA prediction especially when they are transcribed from the intergenic deserts. The other reason is that comparative studies on the sequences of ncRNA have failed to show any healthy

conservation among the known transcripts reported till date. However, studies on a handful of functional ncRNAs indicate that they carryout common functions via conserved secondary structures, indicating that despite having no sequence similarity they seem to harbor conserved secondary structures. Even though a large number of genomes have been sequenced, the number and diversity of ncRNA-encoding genes is largely unknown, especially due to the incompleteness of the list of various ncRNAs (van Bakel et al. 2010; Farh et al. 2005).

15.2 Classification and Evolution of ncRNA

The existing classes of ncRNAs are in general transcribed by all three possible modes of transcription. The pre-rRNA (28S, 18S, 5.8S) are transcribed by Pol I, and some of the snRNAs and LINEs are transcribed by Pol II where as SINEs, snRNA, 7SL RNA, etc. are transcribed by Pol III transcription machinery. Broadly, ncRNAs can be classified into "housekeeping" and "regulatory" ncRNAs (Morey and Avner 2004). Housekeeping ncRNAs are constitutively expressed and involved in processes like translation, RNA processing, RNA modifications, protein trafficking and genomic stability required for normal cell viability, whereas the regulatory ncRNAs, often expressed in certain specified tissues during different stages of development or in response to an external stimuli and they are comprised of RNAs involved in the process of gene expression/regulation and chromatin organization. The other way to classify ncRNA is based on the size of the functional transcripts as long (9,999–10,000nt), medium (200–999nt), small (24–199nt) and micro (18–31nt) ncRNAs. Noncoding transcripts can also be classified based on the sequence origin as sense or antisense transcripts from the genic, intronic and intergenic region of the genome.

The human genome has approximately 27,161 genes (Flicek et al. 2008) in total, of which about 4,421 are ncRNA genes. Literature on the existing ncRNAs indicates an unequivocal correlation between the rise in the number of noncoding transcripts and the complexity of an organism (Amaral and Mattick 2008). Events responsible for such evolution are by gene duplication, mutation, horizontal transfer and integration of genetic material between different pathogen and host across various phases of evolution. Especially the non-genic deserts are more prone towards such events since the pressure to preserve the functionality of the protein coding genes does not apply to ncRNA coding intergenic regions. Also the drastic mutations can be well tolerated since the constraint for most of the ncRNA is to maintain its secondary structure. Moreover the base change in one strand is often compensated by a complementary mutation across the paired strand. Though ncRNAs in general are rapidly evolving they are earmarked by conservation in their secondary structure and are often found associated with regions spanning promoters, splice junctions, and other regions with specific chromatin signatures in relation to the spatiotemporal expression and subcellular localization pattern (Pang et al. 2006; Bradley et al. 2009).

15.3 Housekeeping ncRNA

rRNAs: Generally eukaryotes have many copies of the rRNA genes organized in tandem repeats; in humans approximately 300–400 rDNA repeats are present in five clusters. All mammalian cells possess two mitochondrial (12S and 16S) and four cytoplasmic rRNA (the 28S, 5.8S, 18S, and 5S subunits) transcribed by RNA polymerase I except 5S rRNA which is transcribed by RNA polymerase III. Most of these rRNAs constitute the active site of ribosomes and also aids in maintaining the fidelity of translation.

tRNAs: tRNAs are the adapter molecules that aid in sequence specific incorporation of various amino acids according to the code present in an mRNA. According to the tRNADB, which is a curated database of tRNA, there are 22 known mitochondrial tRNA genes and 497 nuclear tRNA genes known in humans but the number varies a lot in different organisms (Abe et al. 2011). These genes are found on all chromosomes, except chromosome number 22 and Y chromosome of humans (Lander et al. 2001). tRNAs are transcribed by RNA polymerase III as pre-tRNAs in the nucleus (Dieci et al. 2007) which undergo extensive posttranscriptional modifications. The adaptor function of tRNA lies in its three-dimensional structure wherein one end of the tRNA carries the anticodon that serves as a genetic code to recognize the codon in mRNA during protein biosynthesis. Transfer RNA-like structures (tRNA-like structures) are a separate class of RNA sequences transcribed from the genome of many plant RNA viruses, which have a tRNA like tertiary structure (Crick 1968). These tRNA-like structures mimic some tRNA functions, such as aminoacylation, but only three aminoacylation specificities, valine, histidine and tyrosine have been reported till date (Dreher 2009). Such tRNA-like structures are also known to increase the stability of RNA viruses by encapsulating its RNA genome (Mans et al. 1991). In addition, they act as 3′-translational enhancers (Matsuda and Dreher 2004) and regulators of minus strand synthesis.

tel-sRNAs: Telomere specific small RNAs called as tel-sRNA are found exclusively in the telomeric region of the genome. These small Π-like RNAs are associated asymmetrically to the G-rich strand of telomers which are Dicer-independent, 2′-O-methylated at the 3′ terminus, and conserved from protozoa to mammalian cells. tel-sRNAs were shown for the first time in mouse genome where they aid in the establishment and maintenance of heterochromatin in the telomeric loci (Cao et al. 2009).

tmRNA: The bacterial *tmRNA* has both tRNA-like and mRNA-like function e.g., 10Sa RNA or SsrA. *tmRNA* engages the problematic messenger RNAs and recycles the 70S ribosomes ultimately that incorporates a series of alanine residues which are earmarked for the degradation of those incomplete peptides (Gillet and Felden 2001). For more information about tmRNAs refer to tmRDB, an exclusive database for tmRNAs.

SRP RNA: The RNA component of the signal recognition particle (SRP) ribonucleoprotein complex also known as 7SL, 6S, ffs, or 4.5S RNA, is a universally conserved ncRNA (Rosenblad et al. 2009) that directs the newly synthesized

proteins within a cell to the endoplasmic reticulum either co-translationally or post-translationally thereby allowing them to be secreted.

snRNAs: small nuclear RNAs can be broadly classified into two. Firstly, the Sm-class of snRNA that possess a 5′-trimethylguanosine cap, 3′stem-loop and heteroheptameric ring structure that binds to sm-proteins. These non-polyadenylated snRNAs are transcribed by Pol II and processed by integrator. The processed mature snRNAs finally aid in splicing out introns from the pre-mRNA. Secondly, Lsm-class RNAs that possess a monomethylphosphate cap and a 3′stem-loop with uridine rich heteroheptameric ring that binds Lsm-proteins. Pol III transcribes such Lsm-snRNAs using external promoters and Uridine stretch as terminator (Segref et al. 2001). Almost all Lsm-snRNPs are assembled in the nucleus within the cajal body for a brief period after which they diffuse out in the nucleoplasm till they reach their specific nuclear domains like, perichromatin fibrils and interchromatin granule clusters. snRNPs containing such ncRNAs form the core of the spliceosome which are the catalytic centers for splicing introns from pre-mRNA (Matera et al. 2007). Among the snRNAs U7 snRNA needs a special mention which is involved in the processing of 3′ end of histone genes of eukaryotes which possess a unique stem-loop structure instead of a poly-A tail. However, snRNAs are not just restricted to splicing events alone as they have been shown to regulate transcription, independent of their splicing function. 7SK is one such snRNA which mediates Pol II transcriptional inhibition via its interaction with P-TEFb. Apart from the above mentioned snRNAs there are numerous other snRNAs which carryout important biological functions like, RNA Pol III transcribed snaR-A RNA, Intergenic spacer RNA (IGS RNAs) etc., are discussed under regulatory ncRNAs section.

SmY-RNA: These ncRNAs belong to a Small nuclear class of ncRNAs in nematodes SmY-RNA were disovered in *Ascaris lumbricoides* during the year 1996 (Maroney et al. 1996). Based on the evidence obtained from the studies carried out in a related species i.e., *C. elegans* SmY-RNA is believed to be in complex with the spliced-leader RNA and involved in mRNA trans-splicing (MacMorris et al. 2007).

snoRNAs: Small nucleolar RNAs, as the name implies, are retained within the nucleolus and aid as guide strands for incorporating the specific modification like methylations and pseudouridylations, onto other RNA molecules like tRNA, rRNA, snRNA etc. snoRNAs can be further classified into C/D Box RNAs, H/ACA Box RNAs, composite C/D Box and H/ACA Box RNAs and Orphan snoRNAs (Bachellerie et al. 2002; Samarsky et al. 1998). In general C/D box members guide 2′O-ribose-methylations and H/ACA members guide pseudouridylation. snoRNAs are defined by the characteristic secondary structure formed by the signature sequences which varies slightly in the composite snoRNAs. The composite snoRNA contains both C/D and H/ACA box and are retained in the cajal bodies and hence, named as “scaRNAs” (Jády and Kiss 2001). U85 a typical example of composite snoRNA, functions in both 2′-O-ribose methylation and pseudouridylation of snRNA. On the contrary, there are snoRNAs with unidentified substrates that are grouped under the Orphan snoRNAs. Apart from their function in guiding modifications for maintaining a stable pool of ncRNA, some members are even known to act like

miRNAs with exclusive regulatory functions and hence they are discussed under the regulatory RNAs section.

15.4 Regulatory RNAs

Regulatory RNAs comprise a subset of both long and small mRNAs having gene expression regulatory function. Regulatory ncRNAs especially the small ncRNAs in general, form base pairs with other RNA or DNA and constitute RNA:RNA or RNA:DNA duplexes. These duplexes are recognised by different complexes like RNA induced silencing complex (RISC), RNA induced transcriptional silencing (RITS) or RNA editing enzymes which act to decipher downstream consequences. These cis-acting regulatory sequences are generally found in non-coding regions of mRNAs and pre-mRNAs. Untranslated regions (UTRs) of mRNA generally act as binding sites for some trans acting regulatory RNAs, though they are also known to form secondary structures facilitating binding of regulatory proteins that in turn control stability, function or localization of mRNAs (Gebauer and Hentze 2004; Moore 2005). Splice junctions provide yet another cis regulatory sequences which along with the aid of spliceosomal snRNAs and other components of spliceosome, a ribonucleoprotein (RNP) complex that controls splicing of the primary transcript (Nilsen 2003; Valadkhan et al. 2007). Regulatory ncRNAs are generally categorized in two classes namely the small (<200 nucleotide) and large (>200 nucleotide) regulatory ncRNAs.

15.4.1 Small Regulatory RNA

There are numerous regulatory RNAs that are <200nt long and show unique spatio-temporal expression in comparison to housekeeping ncRNAs. Some of the well charactreised small regulatory RNAs are discussed in this section.

Regulatory Small nucleolar RNAs (SnoRNAs): Some SnoRNAs show tissue specific expression, like tandemly arranged repeated intron-encoded C/D snoRNA genes in the region downstream from the GTL2 gene at 14q32 show brain specificity. These snoRNA genes associate with human imprinted 14q32 domain suggesting their regulatory role in epigenetic imprinting process (Cavaillé et al. 2002). Two other brain specific snoRNAs, HBII-52 and HBII-85 were reported to be absent from the cortex of a patient with Prader-Willi syndrome (PWS), which is a neuro-genetic disease resulting from a deficiency of paternal gene expression, indicating their role in the etiology of PWS (Cavaillé et al. 2000). Further, it was shown that the snoRNA HBII-52 regulates alternative splicing of the Serotonin Receptor 2C. Lack of HBII-52 in PWS patients generate different messenger RNA (mRNA) isoforms which leads to the loss of high-efficacy serotonin receptor, which could contribute to the disease (Kishore and Stamm 2006).

Apart from regulating mRNA transcription which is central for the regulation of gene expression, other biological reactions comprehending gene expression like mRNA turnover, gene silencing and translation are also controlled by ncRNAs like miRNAs and short interfering RNAs. miRNAs and siRNAs are 21–25 nt long RNAs derived from double stranded RNA precursors. Origin of miRNA is endogenous from short hairpin precursor RNAs whereas siRNA are mostly exogenous from double stranded RNAs or long hairpins. These small RNAs regulate gene expression through translational suppression (post transcriptional) and/or mRNA degradation (transcriptional) by perfect/non-perfect match formed between miRNA and target mRNA (Mattick and Makunin 2005; Yekta et al. 2004; Mansfield et al. 2004). siRNA is also known to regulate gene expression by modulating chromatin structure (discussed in next section). MicroRNA genes are generally transcribed by RNA polymerase II generating primary miRNA (pri-miRNA). Pri-miRNAs are several kilobases long and possess stemloop structure. Pri-miRNAs are cleaved by RNase III, enzyme Drosha, containing multiprotein complex, producing ~70-nt hairpin precursor miRNA (pre-miRNA). Pre-miRNA is exported to cytoplasm where it is processed into ~22 nt miRNA duplex by another RNase III enzyme, Dicer (Bushati and Cohen 2007 for review). Dicer along with protein argonaute form a complex triggering the assembly of ribonucleoprotein complex called as RNA-induced silencing complex (RISC). One strand of miRNA gets incorporated into RISC and guides the complex to target RNA for base pairing. In case of perfect match with target RNA it is cleaved and if base pairing is imperfect and the binding is strong enough to hold, then the translation is repressed. Major mode of action of animal miRNA involves translational repression rather than RNA degradation unlike the plant miRNAs (Millar and Waterhouse 2005a). Target recognition of miRNA mainly depends on the stringency of base pair match at the 5′ end of miRNA called as the "seed region". Nevertheless, when the 5′ sites are dominant, it can function with or without 3′pairing support. In case of insufficient 5′ pairing in some miRNAs, the 3′ compensatory sites play their part by strong pairing with the seed region sequence.

esiRNAs: Initially endogenous siRNAs (esiRNAs) have been detected only in organisms that possess RNA-dependent RNA polymerases (RDRPs) and absent in others which lack endogenous dsRNA (Millar and Waterhouse 2005a, b). However, other sources of dsRNAs including long hairpin structures generated from the palindromic sequences and dsRNAs generated by the annealing of complementary RNAs that are synthesized by two opposing transcription units in the same loci. Such dsRNAs have now proven to be the source of esiRNAs in both *D. melanogaster* and mice (Watanabe et al. 2008; Tam et al. 2008). In Drosophila esiRNAs have been shown to play important role in the formation of heterochromatin within the somatic tissues (Fagegaltier et al. 2009). esiRNAs have also been implicated in suppressing the expression of mobile genetic elements. Mice deficient for Dicer showed elevated expression of only certain transposable elements which are believed to be affected by the esiRNA pathway but the exact mechanism is yet to be discovered (Nilsen 2008).

Viral miRNAs: These are the viral transcripts that are generally employed in processes like immune recognition, cell survival, angiogenesis, proliferation and cell

differentiation upon infection of the host cells (Pfeffer et al. 2004; Gottwein et al. 2007; Grey et al. 2010). A recent review on viral miRNAs has listed the known viral miRNAs from different viral species (Plaisance-Bonstaff and Renne 2011). miRNAs in general show a higher degree of conservation but viral miRNAs on the other hand shows very poor sequence homology between viruses. Viral miRNAs targets only a small sub population of viral transcripts and obviously they target the majority of host mRNA transcripts thereby regulating their expression to substantial level to create a conducive, microenvironment for their survival and proliferation of viruses (Grey et al. 2010). Virus encoded miRNAs are known to act as suppressors of RNAi, modulating the host miRNAs and also incorporates epigenetic changes in the host which may aid in the viral oncogenesis (Scaria and Jadhav 2007).

Y RNAs: These are small noncoding RNAs that function as integral part of the Ro RNP. The Ro RNP was discovered by Lerner et al. in systemic lupus erythematosus patients. So far four Y RNA species have been discovered in humans namely hY1 (hY2 is a truncated form of hY1), hY3, hY4, and hY5 RNAs ranging in size from 83 to 112 nucleotides (Hendrick et al. 1981). Y RNAs are expressed in all vertebrate species studied (Perreault et al. 2007). Among the invertebrates Y RNA orthologues have been reported in *Caenorhabditis elegans* (Van Horn et al. 1995; Boria et al. 2010) and *Deinococcus radiodurans* (Chen et al. 2000), but no orthologues in yeasts, plants, or insects. In Deinococcus, Y RNAs are reported to be involved in 23S rRNA maturation (Chen et al. 2007). while the human Y RNAs (hY RNAs) aid in the process of chromosomal DNA replication which ultimately ensures a completely semiconservative mode of replication throughout the genome. They have been implicated in either the initiation steps to establish an active replication forks or for elongation steps during DNA replication fork progression (Christov et al. 2006). Recently hY RNAs were shown to be even overexpressed in solid tumours, that aids in cell proliferation (Christov et al. 2008). Nevrethless the cause and consequences are not yet completely deciphered.

TSSa-RNAs: Transcription start site–associated RNAs as their name suggests are transcribed either as sense or antisense transcripts from region flanking the active promoters, with peaks of antisense and sense short RNAs peaking between nucleotides −100 and −300 nucleotides upstream and 0 to +50 nucleotides downstream of TSS, respectively. In yeast such TSSa-RNAs are called as cryptic unstable transcripts (CUTs) and stable unannotated transcripts (SUTs) (Neil et al. 2009; Wyers et al. 2005; Xu et al. 2009). TSSa-RNAs are 20–90 nt (Seila et al. 2008) in length and have been proposed to aid in maintaining poised chromatin state at the promoter regions for downstream transcriptional regulatory steps. The transcription initiation factors, RNAPII and the K4-trimethylated histone H3, occupy the same position over the chromatin where TSSa-RNA; whereas, K79-dimethylated histone H3, is located downstream of TSSs. Recently, a long promoter associated ncRNA (pncRNA) has been identified which repress the protein coding transcripts in cis via an RNA binding protein called TLS (Translocated in liposarcoma) that mediates transcription repression through HAT inhibition. Refer Sect. 15.4.3 for more details.

vRNAs: vault RNAs are integral part of the vault particles that were discovered as a vault ribonucleoprotein complex implicated in multidrug resistance and intracellular transport. Generally these are 100 bases long and transcribed by Pol III. vRNAs via a DICER mechanism generate small vault RNAs (svRNAs) that act like miRNAs in downregulating the expression of CYP3A4, an enzyme essential for drug metabolism (Persson et al. 2009).

15.4.2 Long Regulatory ncRNA

Long Regulatory ncRNA (lncRNAs) as mentioned earlier are greater than 200 nt long and both polyadenylated, and nonpolyadenylated transcripts have been reported. Apart from intronic and intergenic (linc RNAs) lncRNAs, they are also encoded from genomic regions enriched with repetitive elements, such as telomeric repeats (TelRNAs), long terminal repeat retrotransposon elements (LINE RNAs), and short interspersed nuclear elements (B2 RNA). lncRNAs often overlap with, or intersperse between the protein-coding and noncoding transcripts. Promoter-associated transcripts, such as promoter-associated long RNAs (PALRs) and promoter upstream transcripts (PROMPTs) have been recently added to the growing list of lncRNAs. Often PROMPTs overlap with PALRs in terms of the size and the distance from promoter. Also they resemble the cryptic unstable transcripts (CUTs) seen in yeasts (Neil et al. 2009). The major roles of most of the lncRNAs are implicated in transcription regulation by altering the enhancers, promoters and other regulatory regions of a gene. This is achieved either by modulating the chromatin structure around these loci or by directly binding to the transcription factors associated to these elements. In general most of these lncRNAs seem to act in a gene-specific manner and recent evidence that lncRNAs themselves may have enhancer activity was suggested by a handful of studies which still remains open for further investigation (Mondal et al. 2010; Ørom et al. 2010).

lincRNAs: The large intergenic non-coding RNAs (lincRNAs) are one among the largest members of lncRNAs which are evolutionarily highly conserved (Guttman et al. 2009). HOTAIR, was the the first lincRNA, identified by Rinn et al. (2007), showing that HOTAIR could influence gene expression in trans by binding PRC2 and targeting it to the HOXD cluster, thereby silencing target genes in HOXD cluster (Rinn et al. 2008). More than 8,000 lincRNAs are known to exist and are well conserved across mammals (Rinn et al. 2008). They are involved in diverse biological processes, like cell-cycle regulation, immune surveillance and in the maintenance of stem cell pluripotency. Often lincRNAs associate with repressive chromatin modifying complexes hence, act as repressors in transcriptional regulatory networks. The typical example being the p53 mediated global gene repression via the lincRNA-p21 triggering apoptosis by recruiting the hnRNP-K on to the defined set of p53 responsive genes (Huarte et al. 2010). Sabine Loewer et al. later described the role of lincRNA in reprogramming events during derivation of human iPSCs

which is presently being described as lincRNA-RoR for 'regulator of reprogramming' (Loewer et al. 2010). These observations indicate that ncRNA has wide reach in regulation of various biological functions.

Totally intronic ncRNAs (TIN): E.M. Reis et al. identified the transcribed intronic ncRNAs (Reis et al. 2005) which are lncRNAs of approximately 0.6–2 kb in length. Later Helder I Nakaya et al. based on the *in silico* predictions available on data sets in different ncRNA databases and using the combined intron/exon oligoarrays they were able to point the intronic regions as key sources of potentially regulatory ncRNAs (Nakaya et al. 2007). They showed that TINs have tissue-specific expression signatures for human liver, prostate and kidney. The antisense TIN RNAs were transcribed from introns of protein-coding genes which are reported to be enriched in the 'Regulation of transcription' Gene Ontology category. Intronic RNAs are believed to regulate the abundance or the pattern of exon usage in protein-coding mRNAs. It has been proposed that TINs regulate the corresponding protein coding genes through transcriptional interference at promoters or through the epigenetic modulation of the chromatin architecture (Louro et al. 2009).

T-UCRs: David Haussler et al. (Bejerano et al. 2004) discovered a group of highly conserved transcripts called T-UCRs (Transcribed Ultra Conserved Regions) which do not code for any protein. There are about 481 such transcripts longer than 200 (bp) with 100% identity between the orthologous regions of the human, rat, and mouse genomes. Since these UCRs are often located at fragile sites in the chromatin and also associated to the genomic regions involved in cancers it is not surprising to link T-UCRs with tumorigenesis. It is also known that some of the UCRs' expression is regulated by microRNAs abnormally expressed in human chronic lymphocytic leukemia, and the inhibition of UCR which is overexpressed in colon cancer could even induces apoptosis (Calin et al. 2007). T-UCR expression landscape in neuroblastoma suggests widespread T-UCR involvement in diverse cellular processes that are deregulated in the process of tumourigenesis.

PROMPTs: In mammals certain long, unstable promoter upstream transcripts (PROMPTs) initiate bidirectionally ~0.5–2.5 kb upstream of transcription start sites that are longer than the TSSa-RNAs (Preker et al. 2008). This class of RNA often overlaps with another class of bidirectional promoter-associated long RNAs known as PALRs which are longer than 200 nucleotides (Kapranov et al. 2007) and are distinct from PROMTs. Interestingly, siRNA targeted to promoter upstream regions often resulted in transcriptional gene silencing. Given that promoter upstream regions associated with bidirectional transcripts, siRNA could have mediated transcriptional silencing via promoter associated transcripts targeting to RNAi pathway (Han et al. 2007). However, the functional link between the expression of PROMTs and PALRs with cognate genes is not yet clear.

GRC-RNAs: A polypurine triplet repeat-rich lncRNAs, designated as GAA repeat-containing RNAs, are ~1.5 to ~4 kb long and localize to numerous intra nuclear punctate foci that associate with GAA.TTC-repeat containing genomic regions. These foci drop in number with more differentiation of the cell type. GRC-RNAs are components

of the nuclear matrix and interact with various nuclear matrix-associated proteins. In mitotic cells, GRC-RNAs localize to the midbody. The interesting part of GRC-RNA foci is that the number increases during cellular transformation (Zheng et al. 2010).

eRNAs: RNA polymerase II binding was noticed over 25% of the gene enhancers which later turned out that these occupancies were not mere landing pads rather they were more of transcription foci for the novel class of ncRNAs without polyadenylation called the Enhancer RNAs (eRNAs) (Kim et al. 2010; Ren 2010). eRNAs synthesis requires a functional promoter but the requirement of other general transcription factors or the mediator complex proteins is yet to be identified. The expression of eRNAs in the enhancer regions generally correlate with the gene activity of neighboring promoters, indicating that these transcripts may be necessary to activate the nearby promoters either by facilitating the formation of more open chromatin or via promoting enhancer promoter communications. Currently RNAi strategies are employed to decipher the precise mechanism of this class of regulatory ncRNAs.

mlncRNA: The mRNA-like ncRNAs are transcribed by Pol II and poladenylated at 3′ and capped at 5′ ends. Most of the members are known to be dysregulated in expression during the pathogenesis of multiple human diseases but their functional roles are yet to be assigned. Studies done so far strongly suggest that their expression is tightly regulated to specific subcellular compartments of specific tissues like brain but the exact role of these RNAs are not known (Inagaki et al. 2005; Jiang et al. 2011).

15.4.3 Small and Long Noncoding RNAs in Transcription Regulation

ncRNAs modulating transcription are abundant and were first to be discovered. Noncoding RNAs as transcriptional regulators target different components of transcription. Mostly such RNAs act in cis or trans and target general transcription factors, RNA polymerase, transcriptional activators or repressors. Here we are providing a few examples of ncRNA which regulate different steps of transcriptional process:

Bacterial 6S RNA: The *E. Coli* 6S RNA is one among the first ncRNAs to be discovered. About four decades ago 6S RNA was sequenced. It is 184 nucleotide long RNA having a conserved secondary structure containing largely double stranded and a central single stranded bulge. 6S RNA forms a stable complex with active polymerase tangled with promoter specificity factor σ^{70}. *E. Coli* 6S RNA was shown to interact with RNAP-σ^{70} complex but not with free σ^{70}, thereby suppressing transcription (Trotochaud and Wassarman 2005). Interestingly, this repression of transcription was true for only a subset of promoters, as 6S RNA can activate transcription at promoters requiring Enzyme- σ^{S} complex (E- σ^{S} is required for survival

during stationary phase), indicating that 6S RNA regulates transcriptional process at multiple levels. Secondary structure of 6S RNA is essential for its activity and notably, single stranded bulge region was found to be critical for its RNAP binding and transcription modulation activity. Furthermore, 6S RNA structure mimic open promoter complex structure seen during transcriptional initiation (as shown in Fig. 15.1a) and thus proposed to inhibit transcription incorporating competition between promoter DNA and the E- σ^{70} (Barrick et al. 2005).

Mouse B2 RNA: B2 RNA is RNAP III encoded transcript, which is transcribed from short interspersed elements (SINE) of mouse genome and it represses RNAP II transcription in response to heat shock (Allen et al. 2004). B2 RNA is 178 nucleotide long and its expression increases many fold upon heat shock. B2 RNA interacts with a RNA docking site on RNAP II and assembles into the preinitiation complex at the promoter disrupting critical contacts between RNAP II and the promoter DNA, thereby inhibiting initiation of transcription (Espinoza et al. 2004). B2 RNA mediated RNAP II transcription repression shows promoter specificity. Recent investigations have explored the mechanisms underlying the B2 RNA mediated repression of RNAPII dependent transcription and found that B2 RNA targets early steps of transcription initiation like the Ser 5 phosphorylation by TFIIH (Espinoza et al. 2007). B2 RNA blocks CTD phosphorylation by TFIIH, only when RNAP II is in a transcriptionally repressed complex over the promoter DNA in an open state (Fig. 15.1b shown in green) prior to the formation of closed (Fig. 15.1b shown in yellow) complex (Yakovchuk et al. 2011).

7SK RNA: The human 7SK RNA is an abundant (2×10^5 copies/cell) evolutionarily conserved nuclear RNA of 331 nucleotides and is transcribed by RNAP III (Murphy et al. 1987 and Zieve et al. 1977). 7SK RNA controls RNAP II elongation by modulating the activity of transcription elongation factor P-TEFb (Nguyen et al. 2001). P-TEFb activates transcriptional elongation by phosphorylating C-Terminal Domain (CTD) of RNAPII. P-TEFb is a heterodimer comprising CDK9 and cyclin T1. In addition to general elongation factor, P-TEFb also functions as an HIV-1 Tat-specific transcription factor. P-TEFb interacts with Tat and the transactivating responsive (TAR) RNA structure located at the 5′ end of the nascent viral transcript thus stimulating HIV-1 transcription. 7SK RNA binds to P-TEFb and represses transcription by abrogating its kinase activity. Association of P-TEFb and 7SK RNA is found to be reversible as ultraviolet irradiation and actinomycin D treatment disrupted P-TEFb/7SK RNA complex which can restore transcription (Yang et al. 2001). Further studies showed that inactivation of P-TEFb by 7SK RNA requires their association with other proteins namely MAQ1/HEXIM1 (hexamethylene bisacetamide-induced protein 1) which form the essential components of 7SK RNP. HEXIM1 was shown to inhibit P-TEFb in a 7SK-dependent manner while 7SK serves as a scaffold to mediate the HEXIM1:P-TEFb interaction (Fig. 15.2b) (Yik et al. 2003; Michels et al. 2003, 2004). A recent investigation has demonstrated that 7SK interacts with chromatin with high affinity (Mondal et al. 2010). The latter observation is consistent with the suggestion that 7SK by interacting with the chromatin serves as a scaffold for recruiting HEXIMI:P-TEFb proteins thereby inhibiting transcriptional elongation.

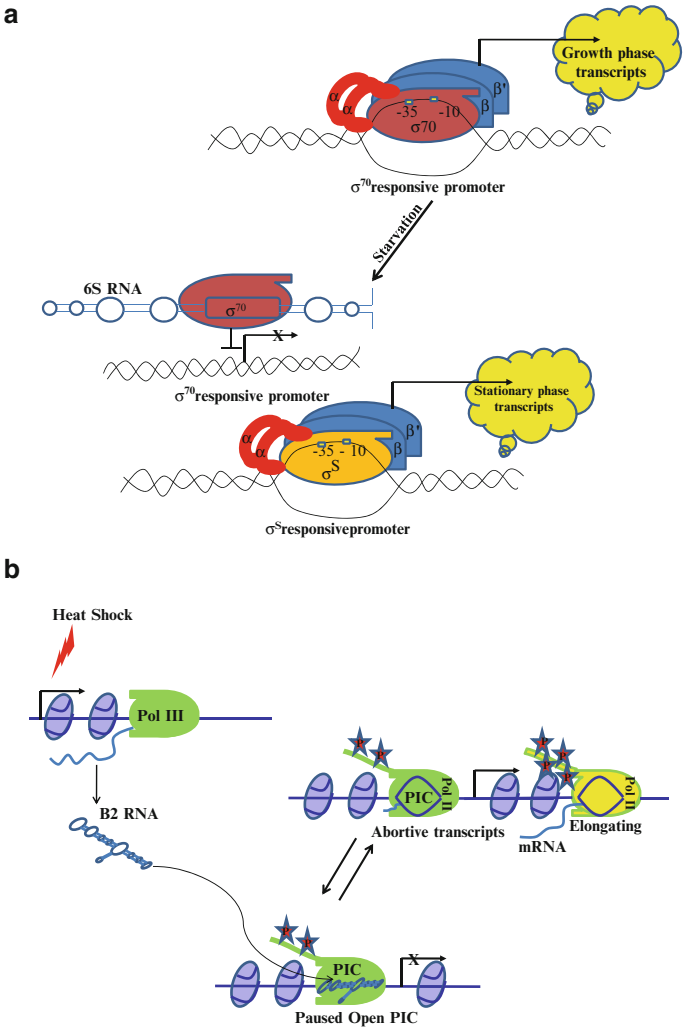


Fig. 15.1 (**a**) 6S RNA mimics open promoter complex. 6S RNA targets the specificity factor σ70. in E. Coli during stationary phase and sequesters from the active polymerase complex and but not free σ70 and hence, blocking transcription during stationary phase. On the other hand during stationary phase 6S RNA activates transcription at promoters requiring Enzyme- σS complex essential for the survival of bacteria. (**b**) B2RNA docks with RNAP II Preinitiation complex and blocks transcription initiation. In response to heat shock, B2 RNA is transcribed by RNAP III which binds RNA docking site of RNAP II within the paused open preinitiation complex over the promoter prior to the formation of closed complexes. This event blocks the critical contacts between RNAP II and the promoter DNA, and also represses the CTD phosphorylation (depicted in *red* stars: 2 stars and 4 stars:) by TFIIH thereby inhibiting the initiation of transcription by RNAP II

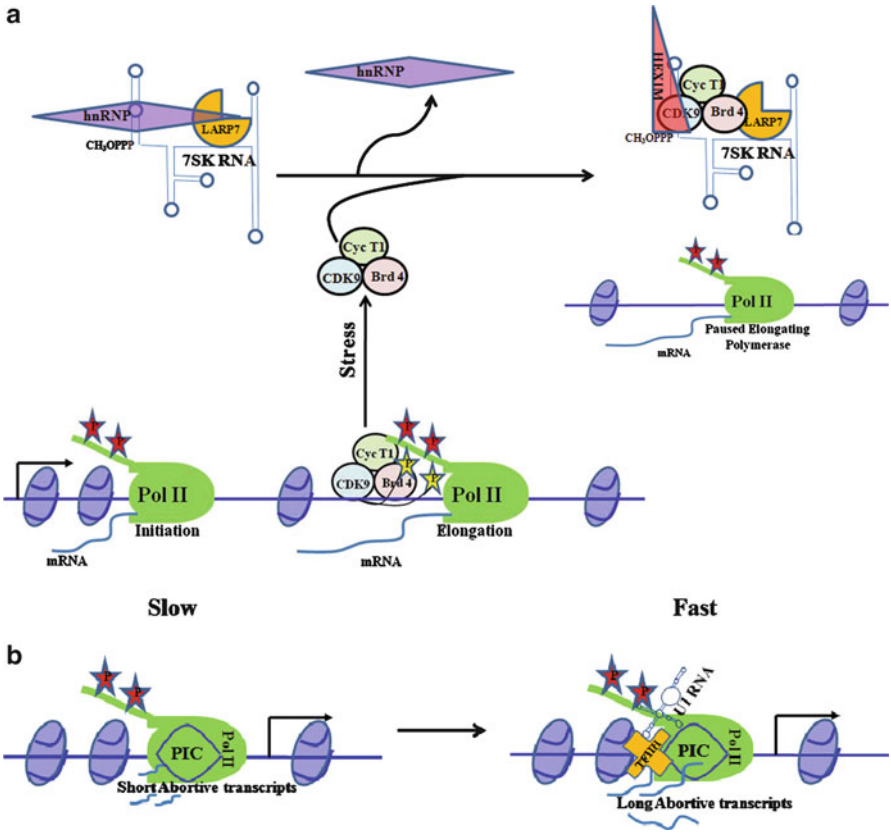


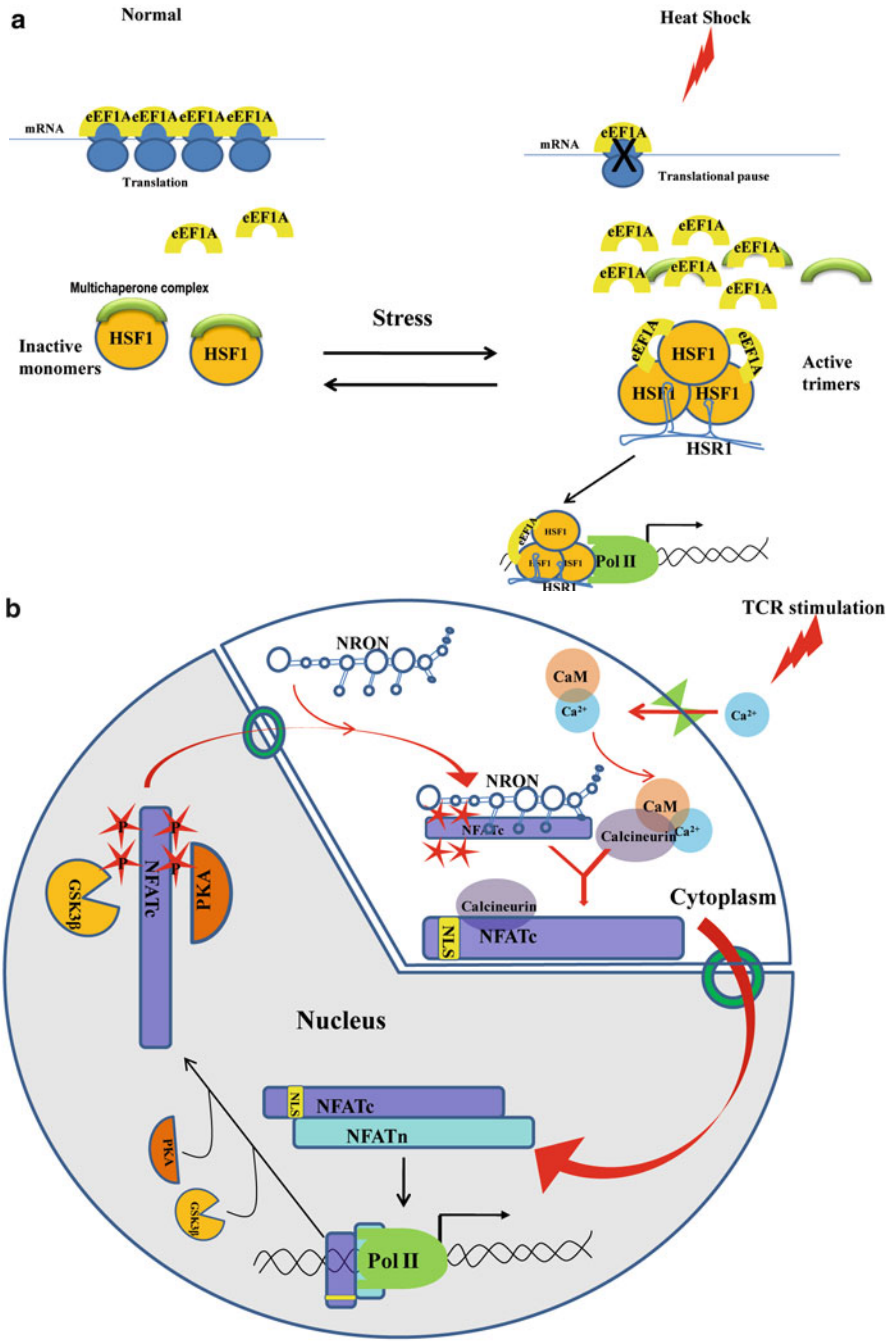
Fig. 15.2 (**a**) 7SK RNA facilitates HEXIM mediated inhibition of P-TEFb. P-TEFb activates transcriptional elongation by phosphorylating (depicted in *yellow* stars) C-Terminal Domain (CTD) of RNAP II. P-TEFb consists of a kinase CDK9 and cyclin T1 heterodimer along with Brd4. Upon stress, the 7SK snRNP is released from hnRNP complex and binds to P-TEFb thereby abrogating its kinase activity and repression of transcription elongation. This inactivation of P-TEFb by 7SK RNA requires their association with other proteins namely HEXIM1 (hexamethylene bisacetamide-induced protein 1) and LARP7 (La ribonucleoprotein domain family, member 7) which form the essential components of 7SK snRNP upon stress. 7SK acts as a scaffold to mediate the HEXIM1:P-TEFb interaction that in turn blocks transcription elongation. (**b**) U1snRNA associates with TFIIH and enhances the transcription initiation rate. U1snRNA binds directly to the cyclin-H subunit of TFIIH and stimulates the kinase activity of TFIIH to phosphorylate C-terminal domain (CTD) of RNAP II, thereby stimulating the rate of initiation

U1snRNA: U1snRNA is approximately 160 nucleotide long ncRNA, transcribed by RNAP II. U1snRNA is one among the five small nuclear RNAs (snRNAs) U1-U6 that exist in snRNPs. These snRNPs facilitate splicing by forming the spliceosome together with many other proteins (Kramer 1996; Burge et al. 1999, for reviews). U1 snRNA has been shown to be associated with one of the general transcription factors TFIIH, thereby influencing transcriptional initiation, a critical regulatory stage of gene expression. Specifically it binds directly to cyclin-H subunit of TFIIH and stimulates kinase activity of TFIIH that phosphorylates C-terminal domain (CTD) of RNAP II. Association of TFIIH with U1snRNA stimulates the rate of initiation (rate of formation of first phosphodiester bond) by RNAP II (Fig. 15.2b). Addition of 5′ splice site adjacent to promoter stimulates reinitiation of transcription in TFIIH dependent manner indicating an important role for U1snRNA in transcriptional regulation by RNAP II apart from its well established role in RNA processing (Kwek et al. 2002).

SRA RNA: The steroid receptor RNA activator (SRA) is approximately 700 nucleotide long natural ncRNA. It exists in ribonucleoprotein complexes and functions as transcriptional coactivators of several steroid-hormone receptors (Lanz et al. 1999). Characterization of distinct RNA substructures within the SRA molecule reveals six RNA motifs critical for coactivation (Lanz et al. 2002). It is not clear whether RNA motifs execute transactivation at the RNA level or in cooperation with RNA binding proteins.

HSR1: Heat-shock RNA-1 (HSR1) is a ncRNA which modulates the activity of heat-shock transcription factor 1 (HSF1) upon heat shock response. In response to heat-shock, HSF1 induces the expression of heat shock proteins. In unstressed conditions, HSF1 exist in an inactive monomeric form and upon activation they acquire trimer formation ability and DNA binding properties. HSR1 and translation elongation factor eEF1A (present as ribonucleoprotein complex) are required for HSF1 activation (Shamovsky et al. 2006). eEF1A when free, is available for interaction with HSR1 and HSF1 which as a complex can initiate the heat-shock response. HSR1–eEF1A complexes when formed would capture HSF1 released from the HSP90 complex and assist its assembly into trimers and/or increase the stability of HSF1 trimers which is considered as the active form, which triggers the transcription of heat shock responsive genes (Fig. 15.3a).

NRON RNA: An RNAi based strategy employed to fish out ncRNAs modulating the activity of nuclear factor of activated T cells (NFAT) led to the identification of NRON RNA (Willingham et al. 2005). The nuclear factor of activated T cells (NFAT) refers to a family of transcription factors important in immune responses. These factors are sensitive to calcium signalling and upon activation calcineurin dephosphorylates NFAT resulting in its nuclear import essential for activating transcription. NRON size ranges from 0.8 to 4 kb based on alternative splicing. NRON represses NFAT activity by regulating its nuclear trafficking probably with aid of various transport factors (Fig. 15.3b). Thus, NRON ncRNA provides example of transcriptional regulation not via RNA-protein interactions or activity modulation of activator but through altering subcellular localisation of the latter.


a
Normal
Heat Shock
mRNA
eEF1A
Translation
Translational pause
Multichaperone complex
Inactive monomers
HSF1
Stress
Active trimers
HSR1
Pol II
b
TCR stimulation
NRON
CaM
Ca2+
NFATc
Calcineurin
Cytoplasm
NLS
Nucleus
GSK3β
PKA
NFATn

pncRNA: Cyclin D1 (CCND1) promoter is associated with lncRNAs (range in size between 200 and 400nt) which are induced in response to genotoxic factors like ionizing radiation (Wang et al. 2008). The CCND1 pncRNA interacts with an RNA binding protein TLS (Translocated in Liposarcoma) and allosterically modify its activity such that this RNA-Protein interactions exert transcriptional repression by blocking the histone acetyl transferase (HAT) activity of CPB/p300 at the repressed CCND1 promoter.

NRSE dsRNA: Neuron-restrictive silencer element double-stranded RNA (NRSE dsRNA) shares sequence complementarity to promoter element that is bound by NRSF/REST (neuron-restrictive silencing factor/RE-1-silencing transcription factor). NRSF/REST is a repressor protein known to silence neuronal genes in non neuronal cells and restricts neuronal gene expression to neurons. NRSE is a small 20 bp double stranded RNA found to activate neural gene expression thus directing neuronal lineage in stem cells (Kuwabara et al. 2004). Interestingly, activation function of NRSE dsRNA is not via base pairing to promoter element with which it shares sequence homology. Rather, it interacts with NRSF/REST and converts this repressor into transcriptional activator. It is proposed that this RNA:protein interaction might prevent association of NRSF/REST with other corepressor proteins thereby switching neuronal gene expression from repressed state in stem cells to activated state in differentiating cells.

piRNAs: Piwi-interacting RNAs (piRNAs) (24–30 nt) are yet another class of small regulatory RNAs whose functions are not fully understood. Piwi family proteins are a subtype of Argonaute proteins and forms RNA protein complex with piRNA. piRNA are found in both vertebrate and invertebrate class of animal kingdom. The best studied function of the piRNA pathway is shown in germline cells where it is involved in transcriptional silencing of retrotransposons (Aravin et al. 2007). Unlike miRNAs and siRNAs, piRNA biogenesis does not involve Dicer or RISC. Not much is known about piRNA biogenesis however, recently it has been shown that a conserved primary piRNA biogenesis pathway that acts selectively on the 3′ UTRs of messenger RNAs having a functional role in gonadal and germline development (Robine et al. 2009).

Fig. 15.3 (**a**) HSR/HSF1/eEF1A trio complex induces transcription of heat shock responsive genes. In normal unstressed condition, HSF-1 exist in an inactive monomeric form along with the multichaperone complex, while the translation elongation factor eEF1A (present in ribonucleoprotein complex) aid in translation process. Upon heat shock, the eEF1A is no more engaged in translation and so they are free to interact with the HSF1 pool and the HSR1-eEF1A complex could assist its assembly into trimers. The ncRNA HSR1 interact with eEF1A-HSF1 trimers to increase their stability and induce the expression of the downstream heat shock responsive genes. (**b**) NRON blocks NFAT shuttling and inhibits NFAT mediated transcription. In normal resting condition NFAT (nuclear factor of activated T cells) remains phosphorylated and associated with the ncRNA NRON as a complex. In response to TCR stimulation the calcium ion entry activates the phosphatase calcineurin. Calcineurin further dephosphorylates NFAT and exposes the NLS, resulting in its nuclear import essential for activating transcription. Further the cytoplasmic pool is restored upon phosphorylation by kinases like GSK3β and PKA

rasiRNA : Repeat associated small interfering RNA (rasiRNA) is considered to be a subclass of piRNA and associate with both the Ago and Piwi Argonaute protein subfamily unlike piRNA which associates only with the Piwi Argonaute subfamily (Girard et al. 2006; Faehnle and Joshua-Tor 2007). Like piRNAs, rasiRNAs are abundant in germline cells and function in silencing transposons and retrotransposons as well as maintaining heterochromatin structure by controlling repeat sequences transcription (Matzke et al. 2004; Lippman and Martienssen 2004; Aravin and Tuschl 2005).

NanoRNA: NanoRNAs are one among the most recently discovered class of functional small RNAs that are believed to affect gene expression through direct incorporation into a target RNA transcript rather than through a traditional antisense-based mechanism. These nanoRNAs were discovered in *Pseudomonas aeruginosa* as 2–4 nt long oligonucleotides that function as primers for initiating transcription from a set of promoters (Goldman et al. 2011). Still the exact molecular events of gene expression, regulatory role remains open for investigation.

15.4.4 lncRNAs in Genomic Imprinting

Genomic imprinting is an epigenetic phenomenon which restricts expression of some genes to one of the two parental chromosomes. So far more than 100 imprinted genes have been identified and most of them are clustered in large chromosomal domains. The allelic expression of imprinted genes is controlled by imprint control element (ICE). ICE is epigenetically modified by DNA methylation and histone modification to regulate the expression of imprinted genes. Only unmethylated ICE is active in inducing repression of flanking genes. ICE attains methylation during gametogenesis and this germline DNA methylation is established by de novo DNA methyltransferases DNMT3A/DNAMT3L (Bourc'his and Proudhon 2008). Subsequent maintenance of methylation at ICE requires maintenance DNA methylatransferase DNMT1 (Hirasawa et al. 2008). In addition, other protein factors (specific for each ICE) also contribute to the establishment and maintenance of ICE methylation (Li et al. 2008). Histone modifications for methylated and unmethylated ICEs are found different. In general, repressive marks like H3K9Me3, H4K20me3 are associated with DNA-methylated ICE and active marks like H3K4me and H3/H4 acetylation with those of unmethylated ICE.

The mechanism by which ICE is proposed to function is either by constituting an insulator region that prevents promoter enhancer interaction or by activating ncRNA transcription. As seen in the *Igf2* imprinted cluster, a methylation sensitive insulator in the ICE regulates its expression. The chromatin insulator protein CTCF (11-zinc finger protein or CCCTC-binding factor) binds to unmethylated the ICE and prevents the communication between the enhancers downstream of the H19 gene and *Igf2* promoters (Kanduri et al. 2000a, b; Bell and Felsenfeld 2000; Hark et al. 2000). DNA methylation of the ICE prevents CTCF binding and allows the enhancer-*Igf2* promoter communication to facilitate its transcription (Kanduri et al. 2001).

In most of the imprinted gene clusters there is at least one macro ncRNA gene. Some of the tested imprinted macro ncRNA have been shown to be indispensable for the imprinted expression of whole cluster (Pauler et al. 2007; Braidotti et al. 2004). Macro ncRNAs are transcribed from unmethylated ICE. These RNAs possess some unusual features such as low intron/exon ratio i.e. reduced splicing potential, nuclear retention and accumulation at the site of transcription (Pandey et al. 2008; Braidotti et al. 2004; Terranova et al. 2008; Nagano et al. 2008). ncRNA mode of regulation is seen more common in imprinted gene cluster expression in contrast to CTCF dependent chromatin insulation mechanism. *Igf2r* and *Kcnq1* imprinted clusters have been used extensively to investigate the role of macro ncRNAs in genomic imprinting. *Igf2r* cluster harbours four imprinted genes in about 500 kb region on chromosome 17: one macro ncRNA *Airn* is exclusively expressed on the paternal chromosome and three neighboring protein coding imprinted genes, *Ig2r*, *Slc22a2* and *Slc22a3* expressed only from the maternal chromosome (Brandeis et al. 1993; Stoger et al. 1993; Lucifero et al. 2002). The unmethylated ICE on the paternal chromosome serves as promoter for paternally expressed ncRNA, *Airn* (Antisense Igf2r RNA) that overlaps *Igf2r* in antisense orientation. *Airn* ncRNA is about 108 kb long, unspliced and polyadenylated transcript. Targeted deletion of ICE, comprising *Airn* promoter, resulted in loss of silencing of all three neighboring genes on the paternal chromosome, indicating that *Airn* ncRNA plays important role in gene silencing (Wutz et al. 1997).

Kcnq1 domain is a one mega-base imprinted domain containing 8–10 imprinted protein coding genes, which are exclusively expressed from the maternal chromosome, and one lncRNA *Kcnq1ot1* expressed from the paternal chromosome. Expression of *Kcnq1ot1* on the paternal chromosome is linked to silencing of the imprinted protein coding genes (Fitzpatrick et al. 2002; Kanduri et al. 2006; DiNardo et al. 2006). However, on the maternal chromosome the imprinted protein coding genes are expressed due to silencing of *Kcnq1ot1* ncRNA promoter by CpG methylation. It has been shown that *Kcnq1ot1* itself mediates transcriptional gene silencing through interacting with chromatin remodeling machinery such as PRC2 complex members and G9a. Furthermore they are targeted specifically to imprinted gene promoters in a tissue-specific fashion thereby organizing higher order chromatin structure devoid of RNAP II (Pandey et al. 2008; Terranova et al. 2008).

Several recent studies have linked differential ncRNA expression to developmental and tissue specific expression of imprinted genes. One such study reveals that neurons do not show imprinted *Igf2r* expression due to lack of *Airn* ncRNA whereas, glial cells which express *Airn* ncRNA shows imprinting of *Igf2r* expression (Yamasaki et al. 2005). Placenta is another example of tissue specific imprinted expression. Several studies indicate the direct involvement of *Airn* and *Kcnq1ot1* macro ncRNAs in placental genes silencing. *Kcnq1ot1* physically localise to several silent genes lying away from promoter (Pandey et al. 2008). It also interacts with polycomb group proteins and establishes repressive marks. Similarly *Airn* ncRNA bind to H3K9 methyltransferase and lies in close proximity to silent *Slc22a3* promoter of *Igf2r* cluster. Deletion experiments involving *G9a* and polycomb group proteins *EZH2* and *RNF2* shows loss of placental tissue specific imprinted expression in these clusters (Nagano et al. 2008; Terranova et al. 2008; Wagschal et al. 2008).

15.4.5 lncRNAs and X-chromosome Inactivation

A best known phenomenon involving the lncRNA is X-chromosome inactivation (XCI). XCI occurs in mammalian females to ensure equal X-linked gene products between two sexes. Inactivated X chromosome expresses a ncRNA called the inactive X-specific transcript (*Xist*) that localizes and coats one of the X chromosome in cis and bring about gene silencing by establishing a higher order heterochromatic compartment. Recent studies have shown that *Xist* interacts with polycomb group proteins like *EZH2* which induces repressive marks like H3K27me and aid in gene silencing (Silva et al. 2003; Plath et al. 2003). The mechanism by which these repressive chromatin modifiers are recruited to inactive X-chromosome is unknown. On the active X-chromosome, *Xist* is repressed and its repression is carried out by a long ncRNA, *Tsix* which overlaps *Xist* in antisense orientation (Wutz and Gribnau 2007). *Tsix*, unlike Xist, silences only the *Xist* promoter on the active X chromosome. However, the mechanisms by which *Tsix* specifically regulates *Xist* repression is currently not clear. *Tsix* has also been shown to interact with epigenetic regulators such as polycomb proteins (Zhao et al. 2008) and DNA methyltransferases (Sun et al. 2006) and this interaction has been suggested to be crucial for the *Xist* repression on the active X chromosome.

15.5 ncRNA in Disease

15.5.1 An Overview

A wide variety of diseases have been discovered with altered expression or function of ncRNAs. Dyskeratosis congenita, Spinal muscular dystrophy, Autism, Alzheimer's, miR96 associated Hearing loss and Prader-Willi syndrome are some of the diseases where the small RNPs like snRNAs, miRNAs and snoRNAs are altered. The Sm-class snRNPs are not properly assembled in spinal muscular dystrophy (Selenko et al. 2001), and in dyskeratosis congenita mutations occur in telomerase RNA (Vulliamy et al. 2001). Duplication of snRNA SNORD115 is associated with Autism. In Alzheimer's disease. an antisense lncRNA (BACE1–AS) is implicated in increasing the steady state levels of its sense counterpart beta-secretase (BACE1) gene by enhancing its stability via masking certain crucial regulatory elements through sense and antisense interactions (Faghihi et al. 2008). This results in increased cleavage of amyloid precursor protein into amyloid beta1-42 which is a critical component in Alzheimer's disease. In case of Prader-Willi syndrome the paternal copies of the imprinted *SNRPN* and *Necdin* genes along with a cluster of 48 *SNORD116* coding region are deleted (Cavaillé et al. 2000; Skryabin et al. 2007; Ding et al. 2008). One other disorder where ncRNAs are implicated in the disease etiology is a rare forms of hearing disorder where the miRNA, miR-96 is aberrantly expressed (Lewis et al. 2009).

ncRNAs also mediate changes at an epigenetic level that ultimately contribute to certain disease etiology. In a rare form of β-thalassemia, a translocation juxtaposes distantly located *LUC57L* in close proximity to the α–globin gene *HBA2*. This results in transcriptional read through from the truncated *LUC57L* transcription unit and specific methylation of *HBA2* gene thus causing transcriptional silencing of *HBA2* gene (Tufarelli et al. 2003). BC1/BC200 an mRNA like ncRNA is known to be altered in the fragile X syndrome, where the loss of function of FMRP (fragile X mental retardation protein) occurs due to the absence of BC200 binding where the subsequent loss of translational repression of mRNAs in the post synaptic area of such patients (Zalfa et al. 2005). Another related ncRNA which has ancestral similarity towards BC200 called as Psoriasis-related ncRNA (PRINS) (Sonkoly et al. 2005) that like BC200 possess two Alu repetitive sequences and was implicated in Psoriasis via the down-regulation of G1P3 (Szegedi et al. 2010) but the exact mechanism is still unkown. Recent reports have shown some SNPs within the non-coding regions associated with certain disease conditions but the complex patterns of ncRNA expression makes it particularly difficult to screen such SNPs (Mattick 2009a, b).

15.5.2 ncRNA and Cancer

In the recent past there is an increasing appreciation in exploring the functional link between ncRNA expression profiles and cancer. Genome wide association studies (GWAS) have now shifted their focus towards miRNAs and lncRNAs' expression patterns in various cancers. The evidences of altered ncRNAs are often correlated well with cancers to a great extent due to the statistically valid observations made from different geographic locations and gene pools. For some of the cancers, these ncRNAs presently, serve as markers for the diagnosis and scoring the treatment regime. snoRNAs, UCRs and miRNAs are some of the commonly reported class of ncRNAs used for such purposes in cancers (Galasso et al. 2010a, b, c). Numerous lncRNAs have been shown to be altered in multiple cancers. For further reference on the list of lncRNAs and the associated cancer type refer to table 3 in ref (recently reviewed by Gibb et al. 2011) and table 1 in a review by Mattick (2009a, b). T-UCRs (Transcribe ultraconserved Regions) are a class of ncRNA that have been reported to be altered in cancers like adult CLL, colorectal carcinoma, hepatocelluar carcinoma and few neuroblastomas where these RNAs are currently being used to predict the patient prognosis with greater confidence (Braconi et al. 2011).

15.5.3 ncRNA and Therapeutics

As mentioned above, numerous ncRNAs have been implicated in the molecular pathogenesis of various human diseases, especially in cancer a special set of miRNAs

possess ongenic properties which are named as “OncomiRs”. Targeting these ncRNAs has always been a valid approach to contain such disorders. Unfortunately the existing information on the functional mechanism involving these ncRNAs is incomplete. The major obstacle for this lack of information is the technical difficulties faced by researchers while performing knock-down of the very few ncRNAs that have been distinctly correlated to a disease state using rigorous screening procedures. siRNA based knock-down does not hold good for ncRNAs but the LNA and PNA based AntagomiRs, and the recently developed synthetic ribozyme based enzymes that cleave specific ncRNA population are showing encouraging results. Unfortunately the efficiency of such molecules is poor. Also the delivery of these antagomiRs poses another level of complexity. Currently people are trying to solve the delivery issues using various vehicles like liposome conjugation, cholesterol conjugation, viral vector based infection and other transgenic and nanomaterial approaches (Galasso et al. 2010a, b, c).

15.6 Outlook

The last decade has been a fruitful year for the investigations on noncoding portion of genome, which previously thought to represents a junk portion of the genome. With the development of several high throughput applications such as microarrays and massive parallel sequencing, it is realized that the majority of the noncoding portion of the genome is pervasively transcribed to encode several thousands of small and long transcripts. Though there is a discrepancy as to the extent of transcription across noncoding portion of the genome, the evidence from several independent investigations provides support to the fact that noncoding transcripts are present in several thousands. Early estimates suggest that existence of about 28,000 lncRNAs and their number could grow well beyond the suggested number. Especially when we consider intronic, antisense and promoter associated transcripts. One of the major challenges associated with this huge number is that detailed physical, structural and functional characterization of each transcript. This will enable us to know the extent of transcriptional noise versus functional noncoding transcripts. Unlike protein coding RNA, lncRNA are expressed at very low level, thus posing a problem in functional annotation of lncRNAs. Hence there is a need for technologies to annotate lncRNAs expressed at low levels. Unlike small RNA mediated silencing pathways, lncRNA mediated silencing and activation pathways are ill defined. Base pair interactions primarily define the specificity of small ncRNAs. Given the absence of sequence similarity between lncRNAs and their targets, it is not clear how lncRNAs specifically activate or silence target genes. This is one of the outstanding questions that remain to be investigated. In the recent past, expression profiles of lncRNA in various cancers have been explored to identify potential prognostic and/or disgnostic markers. Like, small RNAs, lncRNAs show distinct expression profiles in various cancers. However, there is not much progress in the treatment of

cancers using ncRNAs as targets. Moreover, the molecular pathways by which lncRNAs induce pathogenesis are not well investigated. Hence the molecular pathways that are affected in response to aberrant expression of lncRNAs need to be well investigated in order to devise better intervention strategies using ncRNAs as targets. Detailed functional annotation of ncRNA transcription across the genome is required in order to realize the potential of ncRNAs in mammalian development and disease.

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Chapter 16
Chromatin Structure and Organization: The Relation with Gene Expression During Development and Disease

Benoît Moindrot, Philippe Bouvet, and Fabien Mongelard

Abstract The elementary level of chromatin fiber, namely the nucleofilament, is known to undergo a hierarchical compaction leading to local chromatin loops, then chromatin domains and ultimately chromosome territories. These successive folding levels rely on the formation of chromatin loops ranging from few kb to some Mb. In addition to a packaging and structural role, the high-order organization of genomes functionally impacts on gene expression program. This review summarises to which extent each level of chromatin compaction does affect gene regulation. In addition, we point out the structural and functional changes observed in diseases. Emphasis will be mainly placed on the large-scale organization of the chromatin.

The genetic material contained in the nucleus has to be transcribed, replicated and repaired all along the cell life, making unlikely the DNA molecules to be haphazardly crammed inside the nucleus. For several decades, evidence has been accumulated showing that the nucleus is highly organized and contains specialized sub-compartments. Several levels of DNA compaction/organization have been depicted: the first and most described one consists in the wrapping of 147 bp around a histone octamer, forming the "bead on a string" or "10 nm" fiber. This elemental level of organization is compacted again in a hierarchical succession of foldings which are all relevant for the understanding of gene expression. In this review, we will mainly focus on the large-scale organization of the chromatin.

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16.1 Local Chromatin Folding and Its Impact on Specific Locus Expression

16.1.1 Local Chromatin Loops

Regulatory elements are crucial for the fine regulation of target genes that can sometimes be located several tens of kb away. To explain this long-range effect above considerable distances, looping out of intervening sequences to bring the regulatory elements in close proximity to the target gene has been proposed to be a common phenomenon.

For instance, looping seems to be a common way to allow enhancer-controlled promoter activation. The best example of such looping events was characterised on beta-globin locus by two independent approaches: Chromosome Conformation Capture (3C) (Tolhuis et al. 2002) and RNA-TRAP (Carter et al. 2002). Both methods (for review on 3C and 3C-like methods see (Simonis et al. 2007)) concluded that the Locus Control Region –LCR– of beta-globin locus contacts the beta-globin gene (Fig. 16.1a). This process is highly specific and dynamic since non globin-expressing cells do not show LCR-gene contact, and since the contacted gene changes according to the fetal-to-adult globin expression switch (Palstra et al. 2003). A promoter-enhancer interaction was also depicted in TH2-cytokine locus (Fig. 16.1b) where the LCR is found in close proximity to IL4/5/13 genes promoter (Spilianakis and Flavell 2004). These interactions are specific of the T-cell lineage and are reinforced when cytokines genes become expressed upon differentiation in TH2-lymphocytes. On this locus, SATB1, STAT6 and GATA3 are essential for both the establishment of the 3D-conformation and the cytokine expression (Spilianakis and Flavell 2004; Cai et al. 2006).

Enhancers and promoters are not the only sequences showing specific contacts. In erythroid progenitors, most of the hypersensitive sites (HS) of the beta-globin locus are aggregated (Fig. 16.1a). It has been proposed that bringing together the LCR and the distal HS creates a local microenvironment called “Active Chromatin Hub” (ACH) in which the expressed globin-gene is recruited (Palstra et al. 2003; Patrinos et al. 2004; de Laat and Grosveld 2003). Many nuclear factors are implicated in the maintenance and the formation of the beta-globin ACH, including EKLF (Drissen et al. 2004), GATA1 and FOG1 (Vakoc et al. 2005), as well as Ikaros (Keys et al. 2008). Such a clustering of regulatory elements has also been described on imprinted Igf2-H19 locus. On this locus spanning over ~100 kb, the gathering of the Differentially Methylated Regions (DMR) leads to two different chromatin configurations on both alleles, keeping the enhancer away from Igf2 promoter on the maternal allele (Fig. 16.1c) (Murrell et al. 2004; Qiu et al. 2008). As seen for the beta-globin locus, several proteins, including CTCF (Kurukuti et al. 2006) and cohesin (Nativio et al. 2009) are required to establish this chromatin conformation.

In addition to their role in gene transcription, chromatin loops have been implicated in the DNA rearrangements occurring in the loci belonging to the immunoglobulin superfamily. The V(D)J rearrangements observed on heavy chain locus, kappa light

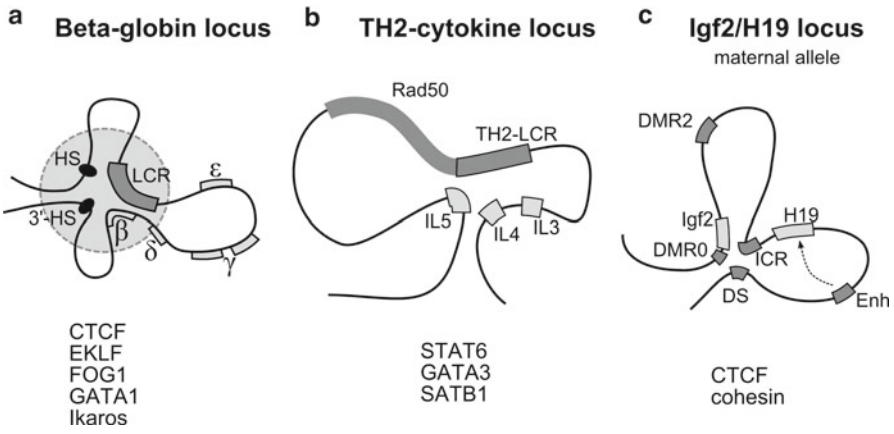


Fig. 16.1 3D-conformation of three loci. Some of the proteins involved in the stabilization/formation of the depicted 3D-conformation are indicated below the drawings. Genes are *light grey* boxes, and regulatory elements are *darker grey* boxes. (**a**) Conformation of beta-globin locus in beta-globin expressing cells. In these cells, the LCR (Locus control region) and the hypersensitive sites (HS) gather, creating a local microenvironment in which the expressed beta-globin gene is recruited (here the β gene). This local microenvironment has been called "active chromatin hub" (depicted in *light grey dashed circle*). Diagram adapted form Patrinos et al. (2004). (**b**) Conformation of the TH2-cytokine locus in the TH2 lymphocytes. The 3′ end of *Rad50* corresponds to a locus control region (LCR) promoting the expression of the cytokine-encoding genes (IL3, IL4, IL5). Diagram adapted from Spilianakis and Flavell (2004). (**c**) Conformation of the maternal allele of Igf2/H19 locus. On the maternal allele, the differentially methylated region 0 (DMR0), the imprinting control region (ICR) and the CTCF binding downstream site (DS) gather. The created loops allow the enhancer (Enh) to activate the expression of H19 gene. On maternal allele, the DMR0, DMR2, ICR and DS are unmethylated (Diagram adapted from Nativio et al. (2009))

chain locus and T-Cell-Receptor locus seem to rely on a local chromatin opening (Oestreich et al. 2006; Cobb et al. 2006) and on the formation of specific chromatin loops facilitating the genomic recombination (Sayegh et al. 2005; Oestreich et al. 2006; Skok et al. 2007).

To sum up, loops of chromatin have been observed on numerous loci and are of functional importance. They maintain in close proximity regulatory elements/gene/sequences ongoing recombination through the binding of specific proteins. This functional organization has been also extensively reviewed elsewhere (Fraser 2006; Schoenfelder et al. 2010a; Sexton et al. 2009; Zlatanova and Caiafa 2009; Kadauke and Blobel 2009).

16.1.2 Targeting to the Nuclear Scaffold

The formation and the maintenance of the loops bring the question of the structural component that could provide a stable framework for the support of the chromatin loops. The nuclear matrix is operationally defined as the residual material remaining from

the nucleus after a high-salt extraction that removes large amount of nuclear proteins. In those high-salt extracted nuclei, the remaining DNA after DNAseI treatment is thought to be strongly linked to the nuclear matrix and to be anchored to a residual skeleton (Razin 2001). These DNA sequences tightly bound to the nuclear matrix have been named Scaffold/Matrix Attachment Regions (S/MARs). The S/MARs are repetitive AT-rich sequences of few hundreds bp (Razin 2001; Liebich et al. 2002), and correspond to portions of DNA with a propensity for curvature (reviewed in Fiorini et al. 2006) and base unpairing (Kohwi-Shigematsu and Kohwi 1990). The S/MARs are often found in introns of large genes, in the boundaries of transcription units and near regulatory sequences (Cockerill and Garrard 1986; Gasser and Laemmli 1986; Käs and Chasin 1987; Keaton et al. 2011).

The identity of the proteins which are of functional importance for the nuclear scaffold is still debated. Nevertheless, some proteins exhibit keys properties allowing them to tether S/MARs on the nuclear matrix. Here we will focus on SATB1, SAF-A, SMAR1 and Topoisomerase II.

SATB1 displays a strong affinity for AT-rich sequences which are over-represented among S/MARs (Nakagomi et al. 1994) and the sequences bound by SATB1 are tightly anchored on nuclear matrix (de Belle et al. 1998). SATB1 also displays a "cage-like" distribution that embraces chromatin-dense regions and forms a network in the nucleus resistant to the high-salt extraction (Cai et al. 2003). All these properties clearly position SATB1 as a potential structural component of the nuclear matrix.

The knockout of SATB1 not only leads to a release of chromatin anchored loops from the matrix but also affects the expression of genes close to SATB1-binding sites. Indeed, at loci where SATB1 should be bound, *satb1*-null thymocytes display a modification of the high-order chromatin structure as well as an aberrant histone post-translational modifications pattern (Cai et al. 2003). This aberrant histone modification pattern is probably due to the ability of SATB1 to recruit in normal cells the chromatin-modification complexes (Yasui et al. 2002). This role SATB1 for anchoring chromatin loops has been clearly established on IL4/5/13 locus (Cai et al. 2006), but also on beta-globin locus (Wang et al. 2009) and MHC class I locus (Kumar et al. 2007). In the last three examples, SATB1 knock-down alters both the high-order folding of chromatin and gene expression.

Taken together, the anchoring property and the ability to recruit complexes that modify chromatin accessibility allow to consider SATB1 (and the nuclear matrix in general) as a structural framework that can simultaneously maintain the chromatin loops and control the expression of looped genes by epigenetic modifications. These findings reinforce the model (yet unproved) in which SATB1 functions as a scaffold where anchored sequences can access to a bound and common regulatory machinery.

The creation on nuclear matrix of a local microenvironment mediating gene transcription has been frequently reported. SAF-A (scaffold attachment factor A), which has also been identified as a component of the nuclear matrix (Romig et al. 1992), has been involved in the recruitment of p300/CBP on the S/MARs of the topoisomerase I locus prior to its transcription (Martens et al. 2002). The authors propose that the acetylation of nucleosomes by p300/CBP on the S/MAR sequences of topoisomerase I locus might create a localized chromatin state favouring the ensuing transcription of topoisomerase I gene.

Tethering to nuclear matrix has also been implicated in the establishment of repressive chromatin states. SMAR1, a MAR-binding protein, has been shown to repress BAX and PUMA expression upon mild DNA damage. This repression is mediated through the recruitment of HDAC1 by SMAR1 on the S/MAR sequences of PUMA and BAX genes. Interestingly, upon severe DNA damage, SMAR1 is sequestered into PML-bodies and no longer binds to the S/MARs of both genes. This alleviates the repression of BAX and PUMA genes and promotes apoptosis (Sinha et al. 2010).

Besides modulating gene transcription, anchoring sequences might also predispose to DNA recombination. First, anchored sequences on nuclear matrix often coincide with nuclear recombination hot-spots (Iarovaia et al. 2004a, b). This might be due to the presence of topoisomerase II, another MAR-binding protein which is a structural and ubiquitous component of nuclear matrix (Adachi et al. 1989). The interferon locus contains two S/MARs and is frequently recombined in various cancers. Interestingly, topoisomerase II is able to tether the two S/MARs of the interferon locus (Eivazova et al. 2009). This physical proximity of the S/MARs combined to the endonuclease activity of the topoisomerase II might favour the illegitimate recombinations frequently observed on this locus. Second, topoisomerase II and the nuclear matrix have also been involved in the caspase-independent excision of DNA during apoptosis (Solovyan et al. 2002). The last two examples illustrate that the roles of the nuclear matrix, although not fully understood, are more diverse than a passive anchoring of chromatin loops.

16.2 Chromatin Folding – From the Surrounding Environment to the Chromosome Territory

It is well established that a selected gene does not show the same expression pattern on its natural context and in an ectopic chromosomal location. Indeed, a gene in ectopic position can be expressed in only a fraction of cells (position effect variegation) or can show lower expression (stable position effect) (Yokoyama et al. 1990; Dobie et al. 1996). These findings are particularly critical for transgenic approaches and one of the strategies developed to overcome these position effects on transgenes generally consists in the addition of insulator sequences (Mlynarova et al. 1994; Alami et al. 2000; Potts et al. 2000). Besides its impact on transgenes expression, the position effects attest of the importance of genomic neighbourhood (or chromatin domain) on gene regulation.

16.2.1 Genomic Neighbourhood

Increasing evidence strongly suggests that the genomic DNA can been segmented in domains exhibiting similar gene expression levels or epigenetic marks (for a recent review, see De and Babu 2010). Indeed, transcriptome data in various species

including human showed that genes are linearly clustered in adjacent groups showing similar levels of expression (Caron et al. 2001; Spellman and Rubin 2002; Versteeg et al. 2003; Mijalski et al. 2005). In human, clusters of highly expressed genes were called "Ridge" and those of weakly expressed genes "Anti-ridge" (Versteeg et al. 2003).

In addition, the profiling of different histone marks also shows that chromatin can be divided in adjacent domains exhibiting either abundance or scarcity in specific histone marks. This has been observed for the content in H3K9me2 (Wen et al. 2009), H2K27me3 (Pauler et al. 2009) and H2AK5ac (Cuddapah et al. 2009). Enrichment in CTCF has been specifically observed at the junctions from high to low content domains. These domains which are cell type-specific and thus might allow a cell-specific gene expression are conserved between mouse and human on orthologs regions (Wen et al. 2009).

The question that we should now address is the functional meaning of this segmentation in domains, and whether it really affects gene expression. Bringing clues to answer this question, Gierman et al. (2007) correlated the expression level of the same GFP transgene with its integration site in 90 different clones. Remarkably, they observed that the expression level of GFP was higher when integrated in Ridge domains (Gierman et al. 2007). Moreover, they analyzed GFP expression according to the expression level of the two closest neighbouring genes (in 5′ and 3′). Strikingly, when integrated in Ridge domains, they found that highly expressed transgenes can be surrounded by either a low- or a high-expressed neighbour. This finding ruled out the possibility that GFP expression only reflects local chromatin configuration directed by the two neighbouring genes and rather emphasized the importance of a domain-wide influence.

In addition to the functional segmentation of the genome by gene expression or histone modifications, a structural segmentation has also been observed. For instance, using FISH, Ridge and Anti-ridge domains are found in different nuclear sub-compartments with very little intermingling (Goetze et al. 2007). This suggests that chromatin fiber is packed in such a way that different functional domains occupy different nuclear locations. Moreover, by coupling 3C-like assays with high-throughput sequencing, the 3D-organization of the human chromatin has been uncovered (Lieberman-Aiden et al. 2009). Using this method, called Hi-C, each interacting partners of any portion of chromatin have been identified with a resolution of 100 kb to 1 Mb. It resulted from this analysis that the bulk genome can be divided in two structural compartments showing mutual exclusions with only few cross-interactions: one correlating with open-chromatin and the other with closed-chromatin. Interestingly, at the mega-base scale, the interaction probability is compatible with an organization in fractal globules segmenting the chromatin in adjacent structural compartments. From both studies, a stimulating concept emerged which states that genomic neighbourhood might be one of the parameters driving the 3D-organization of chromatin fiber.

16.2.2 Gene-Expression in the Context of Chromosome Territory (CT)

In the nuclear space, each chromosome occupies a defined volume which has been called chromosome territory (CT) (Cremer and Cremer 2001). It has been observed that chromosomes are positioned according to their gene-density, with small, gene-rich chromosomes (chr 16, 17, 19, 20, 21, 22) exhibiting a central position in the nucleus whereas gene-poor chromosomes show a more peripheral location. This property tends to be evolutionary conserved (Tanabe et al. 2002) even if some variations have been observed from one cell type to another (Parada et al. 2004). The borders of CTs are not well defined and some intermingling between chromosomes occurs at these junctions (Branco and Pombo 2006). Combined to the non-random radial organization of CTs, the chromosomes intermingling allows specific *in-trans* contacts with neighbouring chromosomes.

Evictions of genes from the core of the spatially constrained CTs have been described. Most of the time, evictions have been correlated with high gene expression or induction (reviewed in Heard and Bickmore 2007; Fraser and Bickmore 2007). For instance the Major Histocompatibility (MHC) class II locus loops out of the chromosome 6 territory while genes become expressed (Volpi et al. 2000; Branco and Pombo 2006); a cluster of more than 40 genes required for keratinocytes differentiation is evicted from the chromosome 1 territory in keratinocytes (Williams et al. 2002); *Hoxb* gene-rich locus from mouse chromosome 11 relocates away from its original CT upon locus activation (Chambeyron and Bickmore 2004; Chambeyron et al. 2005); and a gene-rich cluster of constitutively expressed genes on 11p15.5 frequently shows extraterritorial location (Mahy et al. 2002). In each example, eviction from CT is correlated with high gene expression, either through gene activation (MHC, *Hoxb* and mouse keratinocytes differentiation cluster) or through constitutive high-expression level (mouse 11p15.5 locus). Besides, inhibition of RNA polymerase II decreases the frequency of extraterritorial location (Mahy et al. 2002), which again suggests that gene transcription is the major process driving CT eviction. Nevertheless, gene activation cannot be the only eviction force since for the *Hoxd* locus, a significant looping out has been observed in tail bud but not in limb bud even if both tissues highly express *Hoxd* genes (Morey et al. 2007). Moreover, a *Hoxb1* transgene inserted in *Hoxd* locus can promote an eviction of the CT even if the transgene is not transcribed (Morey et al. 2008). Both findings thus imply that transcription activation and eviction can be at least partially uncoupled.

The intermingling of chromosome territories and the motion of giant chromatin loops outside their original CT allow the establishment of interchromosomal (trans) contacts. A *trans*-interaction has been described in naïve T-cells between Ifng gene (a TH1-cytokine gene, mouse chromosome 10) and the TH2-cytokine genes locus (mouse chromosome 11). Upon differentiation in either TH1-lymphocytes or TH2-lymphocytes, this trans-interaction is lost concomitantly with the expression of the appropriate cytokine genes (Spilianakis et al. 2005). Furthermore, the deletion of a hypersensitive site on TH2 locus both decreases the *trans*-contact and alters *in*

trans the expression program of Ifng, providing functional relevance to this physical interaction. Similar *trans*-interactions have been extensively depicted including between alpha- and beta-globin locus (Osborne et al. 2004; Brown et al. 2006, 2008), between X-inactivation centers (Bacher et al. 2006; Xu et al. 2006, 2007; Augui et al. 2007), between Igf2/H19 and Wsb1/Nf1 loci (Ling et al. 2006), between cMyc and Igh loci after the stimulation of resting B-cells (Osborne et al. 2007), and between H enhancer and olfactory receptor genes (Lomvardas et al. 2006). These chromatin *trans*-associations have been detected by both 3C-like methods and FISH experiments. The major question that remains to be addressed is about the functional relevance of these chromatin *trans*-interactions and whether they do not only reflect topological constraints inherent to chromosome folding. If the long-range *trans*-contacts do actually represent functional interactions, much care should be taken before pointing out the nuclear processes mediating these interactions (as commented in Williams et al. 2010).

The long-range contacts between loci tend to colocalize at specific subnuclear compartments (reviewed in Ferrai et al. 2010). The main nuclear compartments involved are the focal concentration of active RNA polymerase II called "transcription factories" (Cook 1999, 2002; Sutherland and Bickmore 2009). The transcription factories are believed to contain the cellular machinery required for the proper expression of active genes. The observation of active alpha- and beta-erythroid genes sharing the same transcription factory (Osborne et al. 2004; Schoenfelder et al. 2010b) has provided an attractive framework to explain their co-regulation. Nevertheless, proofs still have to be clearly established to demonstrate that an interaction is required for the proper coregulation of alpha- and beta-globin genes. Furthermore, the measured distances between "colocalized" alpha- and beta-gene is significantly above the diameter of a transcription factory, which led other authors to rather propose a localization of erythroid genes at the same nuclear splicing speckle (Brown et al. 2006, 2008).

16.3 Setting Up the High-Order Chromatin Folding During Development

The non-random distribution of CT and of genomic loci described earlier may be further exemplified by the extreme rearrangement of chromatin observed in the rod photoreceptor cells of nocturnal mammals (Solovei et al. 2009). Here, an inverted organization is found with heterochromatin occupying the interior-most part of the nucleus. The chromatin, in a very striking example of sub-cellular, lineage specific evolution, then acts as a lens, increasing photons collection and thus the overall efficiency of the eye. In mice, this peculiar architecture is established during the first month of life after the rod cells has stopped dividing.

This immediately raises the question of the nature of the driving forces able to establish and maintain this organisation during development. The movement of particular loci during development has been studied in *C. elegans* (Meister et al. 2010).

One of the main results of this study is that promoters of tissue specific genes are able to somehow impose the subnuclear position of their loci, bringing them, upon developmental activation, towards an inner position. Different hypothesis may be put forward in this regard. In their study, Meister et al. demonstrate that activation of the gene precedes its movement. It can be proposed that the ensuing modification of chromatin, induced by the recruitment of remodelling machines, helps to tether the activated loci in the interior of the nucleus, for example on speckles or on sites of active transcription. Since activation precedes relocation in this context, subnuclear localisation can only be viewed as one of the mechanisms activating or repressing genes. It should also be remembered that there is not a strict correlation between the position of a locus within the nucleus and its activity. As an evidence, it was indeed shown that only some genes display a significant decrease in transcription when experimentally tethered to the nuclear envelop (reviewed in Towbin et al. 2009).

At a larger scale, and as described earlier in this review, chromosomes with high density of genes tend to occupy a more internal position within the nuclear volume compared to their gene poor counterparts. Koehler et al. carried out elegant series of *in situ* experiments to establish the time during development at which this peculiar organization is set up for the gene-rich chr19 and the gene-poor chr20 (Koehler et al. 2009). Using *in vitro* fertilized bovine embryos, they showed that this gene density correlated positioning is not seen at 4- to 8-cell stages of bovine development, and become observable at the time of the major genome activation, i.e. 8- to 16-cell stage. They showed furthermore that this corresponds to an internalisation of chr19 rather than a displacement of chr20 towards the periphery. This study clearly establishes a correlation between transcription and large scale organisation of the nucleus. It is presently not clear if the movement of a CT is the sum of the elementary movements of each loci linked to the CT, or if a force acts on the chromosome as an entity. These possibilities, though not exclusive, are conceptuality different. To account for the first possibility, we can imagine a scenario in which individual loci will associate to nuclear structures according to, for example, their transcriptional status. This association would be established after the diffusion of the loci within the nucleus and would be stabilised thanks to the affinity of the loci (and, say, its associated transcriptional machinery) towards the nuclear structures. The sum of individual movements will eventually impose the global architecture of the nucleus. In this scenario, no molecular motor are required. Regarding the second possibility, one has to summon ATP, motors, and particular loci where generated forces should be applied.

Indeed, there are different arguments in favour of the existence of force generating, energy-consuming phenomena able to move loci or entire chromosomes. Although not in the context of development, Mehta et al. recently provided data showing that, in primary cultures of fibroblasts, chromosomes were quickly repositioned upon serum starvation (Mehta et al. 2010). This phenomenon is shown to require energy, and seems to be dependant on actin and nuclear myosin 1β. The energy dependence of nuclear organisation may find its source in transcription itself. Consistent with this view are the results of numerical simulations that suggest that entropic effects, due to the interactions of active genes in transcription factories, may be the driving force of non random CT positioning in the nucleus (Dorier and Stasiak 2010, and

references therein). In this context, during development, the modification of the genes transcriptional status would thus affect the position of whole chromosome territory. On the other hand, and because actin is required for chromosome movement, it can be hypothesized that molecular motors more or less specifically rearrange the nuclear structure at different scales. In a recent review, clues in favour of this view have been presented (Gieni and Hendzel 2009).

Clearly, if common themes can be unveiled, much more remain to be fully described to get a better understanding of the establishment and maintenance of the high level of nuclear architecture.

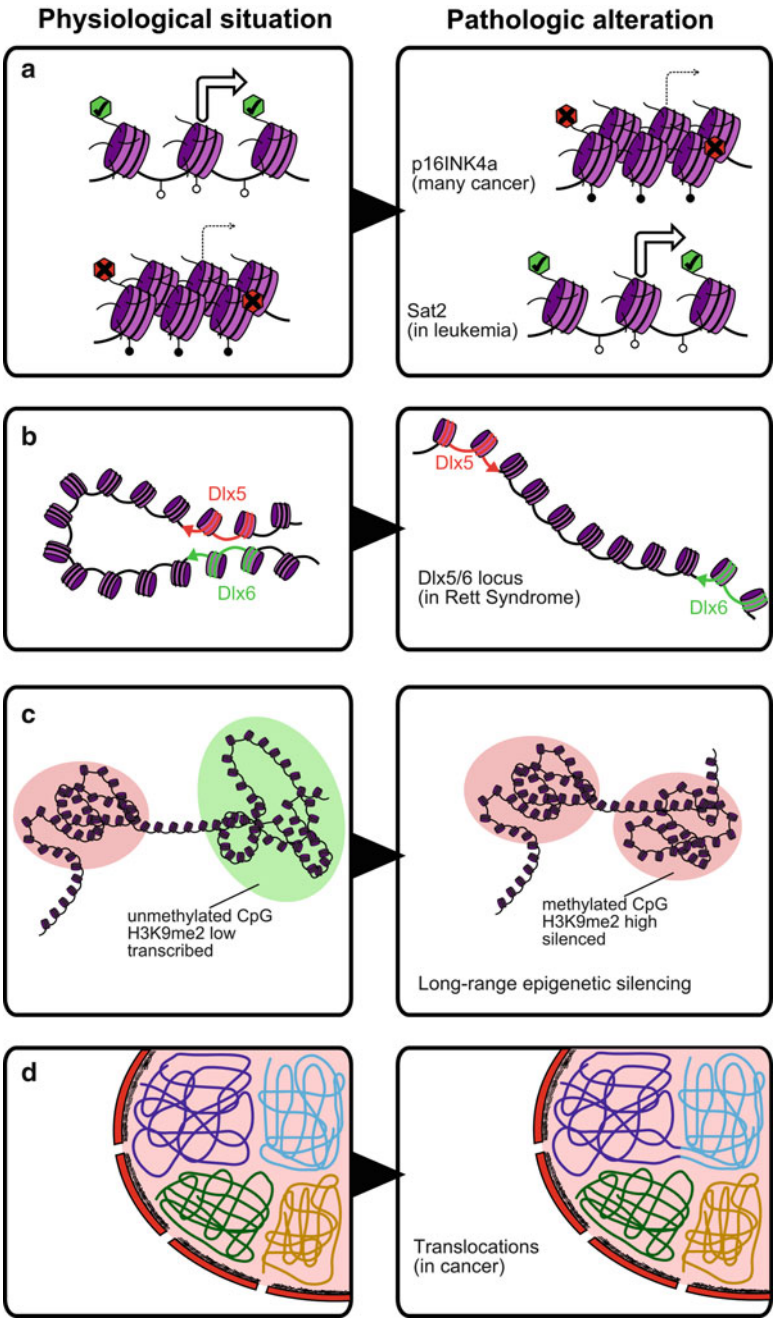
16.4 High-Order Folding of Chromatin and Diseases

Several pathologies have been linked to the epigenetic alteration of specific genes (Fig. 16.2a) and, accordingly, a new and important research field deals with the epigenetic origins and development of diseases. The diseases caused by the mutation of genes involved in epigenetic mechanisms have been extensively reviewed (Portela and Esteller 2010; Allis et al. 2007). As this review is focused on the large-scale analysis of chromatin structure, we will only describe in the following sections how large-scale chromatin conformation can explain/be affected by diseases development (Fig. 16.2).

16.4.1 Spatial Proximity and Translocation Frequency

The Chronic Myelogenous Leukaemia (CML) was one of the first cancers linked to a genetic abnormality, namely a t(9;22) translocation that fuses part of the BCR gene to the ABL gene and leads to the formation of an oncogenic protein (Nowell 2007). Interestingly, in the haematopoietic lineage of healthy donors, BCR and ABL loci are more often close to each other than randomly expected (Lukásová et al. 1997)

Fig. 16.2 Chromatin organization and its alteration in diseases. (**a**) at the nucleosome level: loci can undergo abnormal compaction (like p16/INK4a in various cancer (Herman et al. 1995)) or abnormal decompaction (like satellite 2 in leukaemia (Fraga et al. 2005)), leading to altered expression pattern. The black lollipops indicate methylated CpG and the white ones indicate unmethylated CpG. The tick on green hexagon indicates permissive histone marks whereas the cross on red hexagon indicates repressive histone marks. (**b**) at the chromatin loop level: Some loci (as for instance Dlx5/Dlx6 locus in Rett Syndrome (Horike et al. 2005)) can undergo major changes in the long-range conformation of the chromatin. (**c**) at the chromatin domain level: a whole chromatin domain can see its histone content modified in cancerous cells (2q14.2 locus in colorectal cancer (Frigola et al. 2006)). The depicted long-range epigenetic silencing of a whole chromosome domain is likely to undergo a structural compaction. (**d**) at the nucleus level: the genomic rearrangements occurring in cancers preferentially happen between physically close genomic loci (Lukásová et al. 1997; Osborne et al. 2007; Lin et al. 2009). Here we depict a translocation occurring between two neighbouring chromosomes


Physiological situation
Pathologic alteration
a
p16INK4a
(many cancer)
Sat2
(in leukemia)
b
Dlx5
Dlx6
Dlx5
Dlx5/6 locus
(in Rett Syndrome)
Dlx6
c
unmethylated CpG
H3K9me2 low
transcribed
methylated CpG
H3K9me2 high
silenced
Long-range epigenetic silencing
d
Translocations
(in cancer)

thus increasing the probability of chromatid exchange. Similarly, a close proximity has been observed between MYC locus and IGH locus (Osborne et al. 2007) facilitating the t(8;14) translocation observed in Burkitt's lymphoma.

To get insight into molecular mechanisms, Lin et al. used a prostate cell line and showed that the combination of a testosterone treatment and a genotoxic stress induces chromosomal rearrangements, with some of them often found in prostate cancer (Lin et al. 2009). Using this method, they observed an androgen-dependent interaction between two loci followed by an androgen-receptor mediated recruitment of the Non-Homologous-End-joining machinery to these loci, likely contributing to the observed chromosomal rearrangements. Since the expression of nuclear receptors is tissue-specific, we can speculate that the nuclear receptor-targeted DNA-recombination could explain the tissue-specific chromosome rearrangements observed in different cancers.

To reinforce the idea that the fusion proteins observed in cancer result from chromatid exchange between spatially close loci, ~50% of human endometrial stromal sarcomas express the fusion protein JAZF1-JJAZ1 (resulting from t(7;17) translocation) which confers anti-apoptotic properties (Li et al. 2007). Interestingly, in normal endometrial stromal cells without chromosomal rearrangement, a chimeric mRNA has been observed whose translation results in the JAZF1-JJAZ1 fusion protein (Li et al. 2008). This chimeric mRNA is believed to result from trans-splicing between the jazf1 and the jjaz1 mRNAs. One would speculate that the spatial proximity between both loci might be the key point to explain the trans-splicing in healthy cells and the chromosomal rearrangement in cancerous cells (Fig. 16.2d).

16.4.2 Change in Euchromatin/Heteochromatin Frontiers in Cancer

Epigenetic alterations play a major role in cancer progression. Abnormal DNA methylation pattern was the first epigenetic change described in cancerous cells with a global hypomethylation on repetitive sequences and a local hypermethylation of some usually unmethylated CpG islands (on tumour-suppressor genes). These epigenetic changes were linked to the mutation and/or overexpression of the DNMT proteins and of the methyl-CpG binding proteins and were extensively reviewed (Esteller 2005; Feinberg 2007; Kanwal and Gupta 2010; Berdasco and Esteller 2010; Portela and Esteller 2010).

Regarding the post-translational histone modifications, a common hallmark of cancerous cells seems to be a global loss in the acetylated H4K16 and trimethylated H4K20 (Fraga et al. 2005). These depletions are found on repetitive sequences and might contribute with DNA-hypomethylation to the expression and instability of repeated-sequences that is observed in various cancers (Gaudet et al. 2003; Ehrlich 2005). To our knowledge, the other global maps of histone modification profiles are tissue-specific and can be use as prognosis factors on specific cancer development or clinical outcome (Seligson et al. 2005, 2009). These global changes in histone

modification pattern result from the alteration (both genetic and epigenetic) of every classes of histone-modifying enzymes (for recent reviews, see Kanwal and Gupta 2010; Ellis et al. 2009; Berdasco and Esteller 2010; Portela and Esteller 2010; Chi et al. 2010).

Until recently, DNA hypermethylation found in cancer was thought to be restricted to selected CpG promoters thus affecting the expression level of a single gene. However, Frigola et al. (2006) discovered a 4 Mb genomic locus that undergoes a global silencing in colorectal cancer associated to an increase in H3K9me2 histone mark. This large portion of genomic DNA contains numerous CpG islands which many of them display an abnormal hypermethylation pattern in colorectal cancer. Strikingly, most of the genes of this 4 Mb genomic locus exhibit a lowered expression in cancer even if some of them remain unmethylated (Frigola et al. 2006). This finding indicates that the lower expression of those genes is mainly due to a global silencing of the whole chromatin domain rather than to an iterative local silencing (Fig. 16.2c). Accordingly, this long-range epigenetic repression brings clues towards the functional importance of the genomic neighbourhood / chromatin domains in the comprehension of global gene regulation.

16.4.3 Change in Euchromatin/Heterochromatin Frontiers in Laminopathies

The laminopathies are genetic diseases caused by a mutation in one of the genes coding for lamina proteins, namely *LMNA* gene or *LMNB1/LMNB2* gene. Lamina proteins are principally located at the nuclear periphery that is thought to be a repressive compartment. Lamina proteins interact with chromatin (reviewed in Dorner et al. 2007; Dechat et al. 2008, 2009). Several pathologies have been linked to mutation in lamina genes (extensively reviewed in Worman et al. 2010); some of them associated with major changes in chromatin structure. For instance, in Hutchinson-Gilford Progeria Syndrome (HGPS) that is mainly caused by the C1824T mutation in *LMNA* gene, abnormalities of the nuclear periphery have been reported, including a lobulation of the nucleus and a thickening of lamina (Sandre-Giovannoli et al. 2003; Eriksson et al. 2003; Goldman et al. 2004). In addition, peripheral heterochromatin is progressively lost in an age-dependent manner. This loss is accompanied by a global decrease in H3K27me3 (related to a decrease in EZH2 level), a depletion in H3K9me(1/3) (related to a decrease in Suv39h1 and Suv39h2 levels), as well as an increase in H4K20me3 (Columbaro et al. 2005; Shumaker et al. 2006). In cells expressing this mutant version of LMNA, both H3K27me3 foci and H3K9me3 foci are redistributed throughout the nucleoplasm (Shumaker et al. 2006), leading to the conclusion that the bulk of the chromatin is reorganized.

The autosomal dominant form of Emery–Dreifuss muscular dystrophy (EDMD) due to the R453W missense mutation in *LMNA* gene, another laminopathy, shows similar alteration of the whole chromatin organization with a decrease in H3K27me3 and a decorrelation between H3K9me3 and the dense DAPI-stained pericentric

heterochromatin (Håkelien et al. 2008). Interestingly, myoblasts carrying EDMD mutation fail to differentiate in myotube, and show improper expression of myogenin gene associated with altered histone marks (Håkelien et al. 2008).

Besides the global reorganization of histone marks, cells with mutations in lamin genes exhibit a relocation of selected chromosome territories. For instance, chr18 territory has been shown to exhibit a more internal location in human cells carrying mutation in *LMNA* gene or in mouse cells deficient in lamin B1 (Meaburn et al. 2007; Malhas et al. 2007). Similarly, human chr13 territory position can change in cells carrying a mutation in *LMNA* gene, preferentially toward a more internal position (E161K, R482L or R527H), even if a more peripheral location has also been described in *LMNA* D596N mutant (Meaburn et al. 2007; Mewborn et al. 2010).

Taken together, these findings point out that laminopathies can be associated with major changes in the large-scale structure of the chromatin; and thus shed light on the important role played by lamina proteins as chromatin/nucleus organizer.

16.4.4 The Case of Aging

Similarly to what happens during tumorigenesis, DNA-methylation pattern changes in an age-related manner: aged cells exhibit lower DNA-methylation level except on some specific genes exhibiting a local hypermethylation (reviewed in Fraga and Esteller 2007; Gonzalo 2010). These changes in DNA-methylation pattern likely reflect the impaired control of expression of the DNMT proteins observed in aged fibroblasts: the global loss in methyl-DNA would be related to the loss in the maintenance DNA methyltransferase DNMT1, whereas the hypermethylation might be explained by the overexpression of the *de-novo* DNA methyltransferase DNMT3b (Casillas et al. 2003). In addition to the modifications in DNA-methylation level, aged cells exhibit changes in histone modifications patterns. For instance, while comparing aged and young samples, it has been reported a global augmentation in H3K27me3, H3K79me(1/2), H4K20me3 and a global decrease in H3K36me3, H3K9me3 (Sarg et al. 2002; Scaffidi and Misteli 2006; Wang et al. 2010). In coordination with the altered pattern of histone modifications, HP1 protein and components of the nucleosome remodelling and deacetylase (NURD) complex including HDAC are lost (Scaffidi and Misteli 2006; Shen et al. 2008; Pegoraro et al. 2009), thus contributing to the global rearrangement of chromatin.

A global chromatin rearrangement is also observed in senescent cells, which leads to the formation of the so-called Senescence-Associated Heterochromatic Foci (SAHF). These foci correspond to a form of facultative heterochromatin enriched in H3K9me3, HP1, and macroH2A but depleted in open-chromatin marks (Narita et al. 2003; Zhang et al. 2005; Narita 2007). Cellular senescence is mainly studied in cultured cells, and its link with organism aging thus remains to be clearly established. Nevertheless, since senescent cells accumulate in aged animals (Herbig et al. 2006), we can hypothesize that the formation of SAHF might be an important phenomenon during aging and might reflect the global epigenetic changes associated with aging.

16.5 DNA Transcription in Coordination with DNA Replication

The duplication of the genetic material takes place while genes keep being transcribed. Although we mostly focused on the link between the high-order folding of chromatin and the gene transcription, we will now underline the interrelation between replication and transcription and their common impact on nuclear architecture.

The analysis of the nucleotide content along the genome highlighted local DNA composition asymmetries consisting in A toward T, and G toward C skews. These compositional asymmetries have been widely observed including in mammals and probably result from asymmetric mutation/repair associated with DNA transcription and replication (Green et al. 2003; Touchon et al. 2003, 2005; Brodie Of Brodie et al. 2005). Thanks to a genome-wide analysis of these asymmetries, hundreds of replication origins (ORI) were predicted in mammals (Brodie Of Brodie et al. 2005; Touchon et al. 2005). Interestingly, around the putative origins, genes are abundant, broadly expressed, mainly divergent, and in a context of open chromatin. All these features progressively weaken while increasing the distance from the origin (Huvet et al. 2007; Audit et al. 2009). This striking observation of a non-random organization of genes/chromatin in the vicinity of these predicted replication origins allows one to consider these detected ORI as privileged loci partitioning the genome in linear successive functional units.

In addition to this linear segmentation of the genome, DNA replication is also involved in some aspects of the 3D-organization of the genome. DNA replication and DNA transcription processes do not take place uniformly in the nucleus: both occur at hundreds of distinct discrete sites, called respectively replication and transcription foci (Cook 1999). Those sites can be easily labelled using analogs of (desoxy)ribonucleotides. Replication foci colocalize with replication factors (reviewed in McNairn and Gilbert 2003) and transcription foci with elongating DNA polymerase II (Iborra et al. 1996; Brown et al. 2008), which consolidates the idea that these foci represent the assemblage of replication/transcription machinery (Cook 1999, 2002). According to the classical model, each of those foci contained several sequences undergoing simultaneous transcription or replication, the sequences forming giant chromatin loops anchored on the factory (Berezney et al. 2000; Berezney 2002; Frouin et al. 2003; Chakalova et al. 2005; Sutherland and Bickmore 2009). Thus in these models, both replication and transcription machineries provide the structural basis and acting forces required to organize the genome in 3D (Chakalova et al. 2005; Fraser and Bickmore 2007; Ottaviani et al. 2008). Interestingly, if both replication and transcription are somehow able to drive the 3D-organization of the chromatin, one would expect that they could share structures. It is not fully the case since it has been observed that replication foci from early S-phase do not colocalize with transcription foci. Nevertheless, both kind of foci exhibit a high degree of proximity since it has been shown that the transcription foci mainly localized at the borders of the replication ones (Malyavantham et al. 2008). This observation suggests that the replication and the transcription could be spatially coordinated in the nucleus.

The interconnection between DNA replication and transcription is also well-established by the fact that most of the genes replicated in early S-phase are actively transcribed whereas the late-replicated ones are mostly silenced (Schübeler et al. 2002; MacAlpine et al. 2004; Woodfine et al. 2004, 2005; White et al. 2004). Moreover changes in replication timing during differentiation have been also correlated to changes in nuclear positioning, with early-to-late changes leading to a more peripheral location and a decreased expression level (Hiratani et al. 2008, 2010). It should also be noticed that chromatin assembly is slightly different in early S-phase and in late S-phase. Indeed, plasmids microinjected in early S-phase (respectively late S-phase) are rapidly assembled into a hyper-acetylated (respectively hypoacetylated) chromatin (Zhang et al. 2002), while plasmids which change their replication timing also switch from one chromatin state to the other (Lande-Diner et al. 2009). These findings depict well how replication and transcription can be intimately connected. Furthermore, the self-interacting structural domains described in Hi-C experiment were interpreted in terms of open/close chromatin reminiscent of gene transcription (Lieberman-Aiden et al. 2009). However, those self-interacting structural domains also tightly correlate with the replication timing, better than any other histone marks (Ryba et al. 2010). Taken together, these results put forward the idea that DNA replication can be as important as DNA transcription to organize chromatin at different scales, from the histone modifications landscape to the structure of chromosome domains.

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Part IV
Epigenetics and Disease

Chapter 17
Cancer: An Epigenetic Landscape

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17.1 Introduction

Cancer is not a single disease, rather a group of abnormality generally associated with uncontrolled cell growth. The characteristics of cancer is determined by its tissue of origin. In humans during the development of cancer the tumor tissue acquires several physiological abilities, termed as "Hallmarks of cancer", through which cancer cells overcome the check points of a cell cycle, avoid the immune surveillance system, disobey the growth regulatory signals and induce the assembly of new blood vessels in the tumor. Later these cells become metabolically hyperactive to harness the energy required for maintaining the various cancer hallmarks. However, apart from these cellular characteristics, physiologically cancer growth and progression is significantly dependant on the "tumor microenvironment". All cancers are genetic but a very few are hereditary. Somatic mutations are considered to be the point of initiation. Nevertheless, the fine tuning of cancer progression, more precisely the establishment of a complex network among the genes expressed in a cancer cell is mediated by the epigenetic reprogramming, which could be affected by the tumor microenvironment (Fig. 17.1). In this chapter we shall discuss about the present understanding of the possible contribution of chromatin modifications and remodelling in cancer manifestation.

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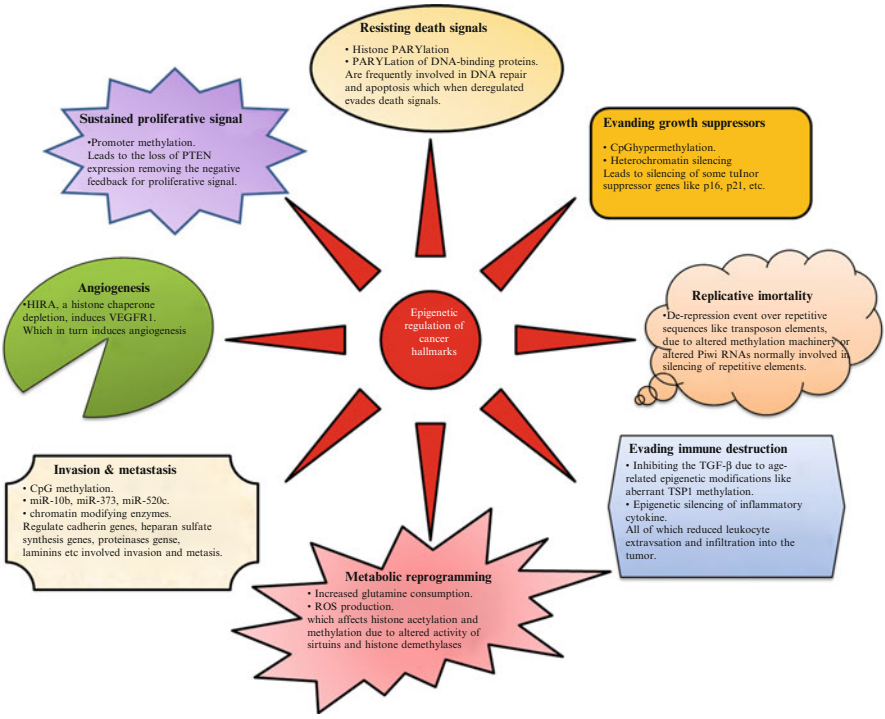

Fig. 17.1 Are alteration of epigenetic network causally related towards the establishment of cancer? Chromatin configuration changes during the acquisition of eight distinguished hallmarks of cancer. These epigenetic alterations are found in both cancer cells as well as tumor associated stromal cells. Here a few representative examples have been depicted

17.2 The Epigenetic Machinery

17.2.1 Histone Post-Translational Modifications

Different posttranslational modifications of histones establish the epigenetic landscape of a functional genome and there by play a key role in the maintenance of cellular homeostasis. In malignancy these histone modifications dramatically alters to support the unusual cancer cell physiology. For example, overproduction the key methyltransferases that catalyze the methylation of either H3-K4 or H3-K27 residues are frequent events in neoplasia. Global reductions in monoacetylated H4-K16 and trimethylated H4-K20 are general features of cancer cells. Recently, it was reported that changes in global levels of individual histone modifications are associated with several cancer. Trimethylation of lysine 27 on histone H3 (H3K27me3) is a target of Polycomb group of proteins, which has been implicated in

the formation of repressive chromatin domains. H3K27me3 spreads over large regions harboring many target genes and negatively regulates transcription by promoting a compact chromatin structure (Francis et al. 2004; Ringrose et al. 2004). In human, H3K27me3 is considered as a prognostic marker in prostate, breast, ovarian, pancreatic and esophageal cancers where the expression levels of H3K27me3 are significantly higher (Füllgrabe 2011). Enhancer of zeste homolog 2 (EZH2) is the catalytic subunit of the polycomb repressive complex 2 (PRC2), which mediates H3K27me3 (Hansen et al. 2008). Overexpression of EZH2 is found in diverse cancers, including prostate, breast, renal and ovarian cancers, as well as glioblastoma multiforme (Füllgrabe 2011). Overexpression of EZH2 has been associated with the invasion and progression of cancers, especially with the progression of prostate cancer (Yu et al. 2007). Generally, EZH2 overexpression in cancer cells seems to result in an EZH2-dependent increase in H3K27me3. However, in some cases of breast, ovarian and pancreatic cancers, no association is drawn between EZH2 and H3K27me3, instead such cases indicate correlation between H3K27me3 and altered HDMs (Wei et al. 2008) JMJD3/KDM6B and UTX (Xiang et al. 2007). Loss of H3K18 acetylation (H3K18ac) is a general marker for active transcription. The H3K18 acetylation has been correlated with poor prognosis in patients with prostate, pancreatic, lung, breast and kidney cancers and loss of this modification is an important event in tumor progression (Seligson et al. 2005; Elsheikh et al. 2009).

Studies have shown that hypoacetylation of histone H4 lysine 12 (H4K12Ac) can be used as predictive biomarkers for cancer recurrence in the prostate (Seligson et al. 2005) and in non-small-cell lung cancer (Barlesi et al. 2007; Van Den Broeck et al. 2008). Global hypoacetylation of H4K12 was even considered to be informative of tumor stage for colorectal cancer (Ashktorab et al. 2009). Histone hyperacetylation has been observed in hepatocellular carcinoma and oral cancer (Bai et al. 2008; Arif et al. 2010).

Recently, increased H3K56ac, as well as upregulated expression of its positive regulator ASF1A, has been observed in many cancers. In humans, H3K56 is the target of both CBP/p300 and hGCN5 (Das et al. 2009; Tjeertes et al. 2009; Vempati et al. 2010) and is found to be deacetylated by different HDACs: SIRT1, SIRT2, SIRT3, HDAC1 and HDAC2 (Miller et al. 2010; Yuan et al. 2009). In addition to this, promoter CpG-island hypermethylation in cancer cells is known to be associated with a particular combination of histone marks: deacetylation of histones H3 and H4, loss of histone H3 lysine K4 (H3K4) trimethylation, and gain of H3K9 methylation and H3K27 trimethylation In another study, the loss of monoacetylated Lys 16 acetylation and trimethylated Lys 20 of H4 were found to be associated with primary tumors and tumor cell lines and they appear to follow pattern in which CpG-island hypermethylation precedes Kirsten rat sarcoma viral oncogene homolog (KRAs) mutations in small colorectal adenomas . Furthermore, loss of CpG island hypermethylation mediated inactivation of tumour suppressor, such as p21/WAF1, has been observed when hypoacetylated and hypermethylated histones H3 and H4 are present at the promoters (Robertson and Jones 2000; Chuang et al. 1997).

17.2.2 Histone Variants

In addition to the altered histone marks, aberrant incorporation of specific histone variants is observed in cancer pathogenesis. The histone cell cycle regulation-defective homolog A (HIRA), a histone chaperone, incorporates lysine 56 acetylated H3.3 over the VEGFR1 gene on triggering angiogenesis within the tumor tissue (Dutta et al. 2010). On HIRA depletion, the induction of VEGFR1 and other angiogenic genes are impaired. A direct link between histone variant expression and cancer development has recently been drawn by Khare et al. They showed that during sequential development of hepatocellular carcinoma, H2A and H2A.1 are overexpressed, whereas H2A.2 is decreased. The increased expression of H2A.1 has been linked to hyperproliferation (Khare et al. 2011). In addition, the histone variant macroH2A appears to suppress the progression of malignant melanoma through the regulation of CDK8 (Kapoor et al. 2010). Furthermore the expression of the human histone variant macroH2A shown to predict lung cancer recurrence and could therefore serve as a useful prognostic biomarker. A strong negative correlation was observed between the macroH2A1.1 and macroH2A2 expression and tumor proliferation rate. Tumors with a low proliferating index shows higher expression of macroH2A1.1 and macroH2A.2, whereas tumors with a high proliferative index often have lower or no expression in most of the clinically aggressive tumors (Sporn et al. 2009).

In a very recent report, it was demonstrated that the expression of macroH2A1.1 is reduced in several types of cancers due to the changes in the alternative splicing of macroH2A1 pre-mRNA mediated by splicing factor, QK1. QKI expression was reduced with the concomitant reduction in the macroH2A1.1 splicing in many of the same cancer types. Furthermore, it was found that the reduction of macroH2A1.1 levels enhances the proliferation of lung and cervical cancer cells with the reduction in the protein levels of PARP-1 (Novikov et al. 2011).

A possible role for H2A.Z in cancer development has first been reported by genome wide gene expression profiling studies. It was shown that H2A.Z was overexpressed in sporadic colorectal tumors. Overexpression of H2A.Z occurred in the later stages of breast cancer progression in a metastatic breast carcinoma (MET) but not in invasive ductal carcinoma (INV). H2A.Z has also been proposed for its role in estrogen receptor signaling and breast cancer progression. Furthermore, overexpression of H2A.Z has been implicated in the destabilization of chromosomal boundaries, which in turn leads to the spreading of repressive chromatin domains and the de novo hypermethylation of tumor suppressor gene promoters in cancer cells (Dalvai et al. 2010 and Santisteban et al. 2011).

Additionally, the highly conserved histone variant H2AX is a sensor of the DNA damage. It rapidly gets phosphorylated by ataxia telangiectasia mutated (ATM) and ATM-Rad3-related (ATR) kinases in the PI3K pathway upon double strand DNA breaks (DSB). It was found that phosphorylation of H2AX and recruitment of repair factors were deregulated in MCF-7 cells compared to normal cell lines like MCF10A cells, indicating the roles of this phosphorylated Histone variant in

maintaining the genomic stability and its perturbations associated with the causal factors for tumorigenesis (Dalvai et al. 2010 and Santisteban et al. 2011).

CENP-A, the centromere-specific H3 variant, is essential for centromeric function and hence chromosome segregation becomes defective in colorectal cancer and it has been suggested that the overexpression of CENP-A is one of the responsible factors leading to aneuploidy (Tomonaga et al. 2005). The possible role of other variants in cancer progression remains to be explored (Henikoff and Furuyama 2010).

17.2.3 Non-Histone Chromatin Proteins and Cancer

Another distinguishing feature of cancerous tissue is the altered expression of histone modifying enzymes due to gene amplification, chromosomal translocations or due to perturbations in the stability of these enzymes and their associated binding partners. For example in leukemic patients after chromosomal translocations involving HATs and HMTs are reported to alter the global histone acetylation and methylation. Especially translocation of HAT and HAT related genes leads to the formation of deleterious fusion proteins. Gene amplification of HMTs and HDMs are a common scenario in solid tumors. The gain of PcG (polycomb) and loss of TrxG (Trithorax) is a common theme in human cancer, demonstrating the oncogenic and tumour suppressive roles, respectively, of these complexes. The oncogenic potential of PcG and its roles in transcription regulation is well established in cancers. Most of these cancers have compromised cellular memory where the stem cell associated genes like Hox genes which are the known targets of PcG and TrxG are reactivated (Mills et al. 2010).

The expression of PRC2 components like EZH2 is upregulated in various cancers such as melanoma, lymphoma, and breast and prostate cancer. EZH2 is presently regarded as an oncogene (Tong et al. 2011). The role of PRC2 in tumour progression is not well distinguished whether PRC2 is required for the process of de-differentiation of somatic cells or it is involved more in the epithelial to mesenchymal transition. PcG-mediated repression is also reversible in cancer cells, as TrxG proteins can override PcG-mediated repression, leading to the reactivation of tumour suppressors.

17.2.4 Chromatin Remodeling and Cancer

Despite intricate packaging, DNA as chromatin, it remains accessible during specific spatio-temporal windows for critical cellular processes such as transcription, replication, recombination, and repair which are facilitated by two classes of enzymes, ATP-dependent nucleosome remodelers and histone modifying enzymes. Chromatin remodelers are macromolecular machines and possess multisubunit components. Chromatin remodeling complexes (CRCs) utilize the energy of ATP

to disrupt nucleosome DNA contacts, move nucleosomes along DNA, and remove or exchange nucleosomes making the DNA/chromatin accessible during cellular processes. In the adult stem cell, deletion or mutation of these proteins often leads to apoptosis or tumorigenesis as a consequence of dysregulated cell cycle control (Hargreaves and Crabtree 2011). ATP-dependent chromatin remodellers can be further divided into families on the basis of subunit composition and biochemical activity, and these families include SWI/SNF, ISWI, INO80, SWR1 and NURD/Mi2/CHD complexes. SWI–SNF chromatin remodelling complexes contain an ATPase subunit (either BRM or BRG1) as well as the non-catalytic subunit (SNF5) that is common to various SWI–SNF-like complexes. Loss of SNF5, BRM or BRG1 has also been associated with human cancer. Malignant rhabdoid tumours (MRTs) a rare but extremely aggressive form of childhood cancer which is caused by biallelic deletion or truncating mutations of SMARCB1 (which encodes SNF5) (Versteege et al. 1998). Among the remodelers, SWI/SNF complexes in particular are emerging as bonafide tumour suppressors (Wilson and Roberts 2011). Several CRCs, most prominently the BAF complex, have been implicated in cancer initiation or progression. Early studies demonstrated that many cell lines have lost both BRG1 and hBRM expression and that introduction of BRG1 or hBRM results in slower or arrested growth.

The vertebrate Mi-2/NuRD complex is a multi-subunit protein complex containing both histone deacetylase and nucleosome-dependent ATPase subunits involved in transcriptional repression. The Mi-2/NuRD complex that is involved in transcriptional repression provides a physical link between histone deacetylation and ATP-dependent chromatin remodeling (Kunert et al. 2009). The MTA (metastasis-associated) proteins represent one class of alternative subunits of the human Mi-2/NuRD complex. The members of this family in human cells are differentially expressed. MeTastasis Associated protein 1 (MTA1), the founder of this family, was discovered in a differential display screen comparing mRNA from rat breast cancer cell line. As the mRNA was expressed at higher levels in cell lines with an increased potential for invasion and metastasis following injection into nude mice, the protein was named as MeTastasis Associated protein 1. Human MTA1 has a discreet correlation between high level expression and invasive growth properties. In addition, a splice variant of MTA1, known as MTA1s, lacks a nuclear localization sequence, is found predominantly in the cytoplasm, and is unlikely to be a component of Mi-2/NuRD. The MTA3 locus in humans also codes for more than one protein based on alternative splicing. In human cancer cell lines, the shorter of the two isoforms (known as MTA3) is more abundant than the longer protein known as MTA3L (Bowen et al. 2004).

Like most chromatin remodelling complexes, INO80 subfamily complexes have been identified as transcriptional regulators. The effect of the INO80 complex on transcription is undoubtedly linked to the function of the transcription factors yin yang 1 (YY1; in mammals) and Pleiohomeotic (in *D. melanogaster*), which are found in INO80 complexes. These transcription factors, which are involved in cell proliferation, differentiation and embryonic development, can serve to specify the genes that are targeted for INO80-mediated chromatin remodelling. In addition the INO80 complex has also been shown to be involved in DNA damage responses.

Recent advances in the fields of DNA repair and chromatin reveal that both histone modifications and chromatin remodeling are important for the repair of DNA lesions, such as DSBs. Recent studies indicate that yeast phosphorylated-H2AX is also required for the recruitment of the chromatin remodeling complex INO80 to DSB sites, thus establishing a link between chromatin remodeling and DNA repair (Morrison and Shen 2009). Furthermore, it was shown recently by the Osley group (Tsukuda et al. 2005) that histone eviction near DSB sites, mediated by INO80 remodeling activity is dependent on MRX (Mre11-Rad50-Xrs2) complex, and a delayed recruitment of the Rad51 repair protein results from defects in histone loss. Taken together, these data suggest that INO80 complex participates in DNA repair pathways by its nucleosome remodeling ability and by regulating the accessibility of repair proteins around the DSB site. Hence, perturbed functioning of INO80 is well correlated to the altered genomic instability that is seen in cancerous cell types.

17.2.5 Non-Coding RNA, Epigenetics and Cancer

17.2.5.1 MicroRNAs (miRNAs) and Cancer

miRNAs are endogenous small non-coding RNAs which regulate gene expression in a sequence specific manner. miRNAs target the 3′untranslated region (3′-UTR) or the 5′-UTR of the target mRNA resulting in mRNA degradation and/or inhibition of translation. miRNAs are involved in most of the biological events, including development, differentiation, cell cycle regulation and metabolism. The full spectrum of miRNAs expressed in a specific cell type (the miRNAome) varies between normal and pathologic tissue, and specific signatures of deregulated miRNAs with diagnostic and prognostic implications. The versatile miRNAs on one hand are regulated by the same epigenetic mechanisms that affect the protein coding genes and on the other hand, a subgroup of miRNAs (epi-miRNAs) targets, directly or indirectly, the effectors of epigenetic machinery such as DNMTs, HDACs and polycomb genes. Thus, miRNAs can also indirectly regulate gene expression by directly regulating epigenetic processes (Pucci and Mazzarelli 2011).

A causal role for miRNAs in cancer was first suggested in 2002 by Croce (Di Croce et al. 2002) and colleagues with the discovery that miR-15 and miR-16, which were located on chromosome 13q14. This region of the chromosome 13 is frequently deleted in chronic lymphocytic leukemia (CLL) patients which are the sole genetic abnormality in such patients. Thus the deletion of miR-15/16 is considered to be a direct cause for CLL. Deregulation of miRNA expression is involved in the initiation and progression of tumorigenesis and has been investigated in almost all kinds of human cancer. Some miRNAs act mainly as tumor suppressors while others have a well-established role as oncogenes, depending upon their target genes. For example, the miR-15/16-1 cluster, that targets BCL2, acts as a tumor suppressor in CLL, whereas let7, acts as a tumor suppressor in lung cancer (Hatziapostolou and Iliopoulos 2011). Oncogenic miRNAs, which target growth inhibitory pathways,

are often upregulated in cancer. MiR-155 and miR-21, are one among the well characterized oncomirs that are induced in several neoplasms. Apart from the above mentioned examples there are multiple other mechanisms which underlie the abnormal miRNA expression in cancers which includes transcriptional deregulation, mutations, DNA copy number abnormalities and defects in the miRNA biogenesis machinery. Epigenetic changes like DNA methylation and histone modifications are also responsible for aberrant miRNA expression. Earlier studies indicate that treatment of different cancer cell lines with the DNA demethylating agent (5-aza-20 -deoxycitidine) and/or HDAC inhibitors is able to alter the expression levels of miRNAs. Several miRNAs (miR-1, miR-124a and miR-127) are under epigenetic control in human cancer due to the fact that they are embedded in CpG island regions and are epigenetically silenced by promoter hypermethylation and histone modifications. Furthermore, it has been proposed that transcription factors can recruit epigenetic effectors at miRNA promoter regions and contribute to the regulation of their expression. (Sharma et al. 2010)

On the other hand, certain miRNAs (epi-miRNAs) are regulators of epigenetic effectors. Epi-miRNAs regulate the expression of DNMT3a, DNMT3b and DNMT1 (miR-29a, -29b, -29c) in lung cancer and AML, RBL2 the inhibitor of DNMT3 genes (miR-290), HDACs (miR-1, miR-140, and miR-449a) in prostate cancer and skeletal muscle tissue and polycomb genes like EZH2 (miR-101) in prostate and bladder cancers. Hence, the involvement of epi-miRNAs and epigenetic regulation of miRNAs introduces additional layers of complexity in understanding the contribution of transcriptional aberration in cancer (Valeri et al. 2009).

17.2.5.2 Long Intergenic Non-Coding RNAs (lincRNAs) and Cancer

Long intergenic non-coding RNAs (lincRNAs) regulate dosage compensation, imprinting, and developmental gene expression by establishing chromatin domains in an allele and cell type specific manner. LincRNAs are intimately associated with chromatin-remodeling complexes, but molecular mechanisms of their functions are still lacking. Accumulating reports of misregulated lncRNA expression across numerous cancer types suggest that aberrant lincRNA expression may be a major contributor to tumorigenesis. Posttranslational modifications of histones recruit DNA-binding proteins and chromatin-remodeling machinery and are often coupled for combinatorial control. For instance, in embryonic stem cells many genes, such as the HOX, that encode developmental regulators are transcriptionally silent but possess bivalent histone H3 lysine 4 (H3K4) and lysine 27 (H3K27) methylation, which are resolved into univalent H3K4 or H3K27 methylation domains upon differentiation. Recently, a lincRNA was shown to coordinate histone modifications by binding to multiple histone modification enzymes (Tsai et al. 2010). lincRNA HOTAIR is known to function as a scaffold for two distinct histone modification complexes. High levels of HOTAIR expression were correlated with both metastasis and poor survival rate. Though, the precise mechanism is not known, it is proven

that HOTAIR reprograms chromatin state to promote cancer metastasis. The MALAT1 gene, or metastasis-associated lung adenocarcinoma transcript 1, like HOTAIR is also associated with high metastatic potential and grave prognosis. MALAT1 is unregulated in a variety of human cancers of the breast, prostate, colon, liver and uterus. Also the MALAT1 locus 11q13.1 often harbors chromosomal translocation breakpoints associated with cancer. Though, numerous findings are reported in the area of linc RNAs their mechanistic correlation towards cancer is open for investigation (Gibb et al. 2011).

17.3 Diet/Nutrition/Environmental Factors Dictating Cancer Epigenetics

Gene versus environment is often the most debated etiology of almost all the diseases. Cancer is not an exception to this argument. In fact, the alarming rise in the cancer population is solely because of the rapidly changing lifestyle that includes what a person consumes, inhales and comes across in his/her life span. This gene: environment interactions occurs constantly in every individual and it is corresponded between the two by epigenetic changes like DNA methylation, histone modifications, histone variant exchange and via other epigenetic signals that ultimately turn off certain genes which are tumor suppressor in function or switch on the expression of oncogenes or rather a combination of both to drive them towards cancer. In this scenario cancer researchers across the globe hypothesize that the changing lifestyle contributes to a considerable amount towards cancer predisposition by compromising the "Allostatic load" of an individual (Knox 2010).

Among the environmental factors diet of an individual has a major impact on cancer susceptibility. Recent reports claim that diet taken by parents can influence the epigenetics of their offspring especially the DNA methylation imprint (Wu et al. 2004; Dolinoy et al. 2006). Histone PTMs contributes to a great extent in the epigenetic signaling and the metabolite source for these modifications is dependent on the cellular metabolism. This in turn is maintained by the dietary input of essential nutrients that gives rise to the metabolites required for chromatin modifying enzymes. Often because of this inherent connection diet influences epigenetics from the very beginning of life and deregulated metabolism is one of the important causative for altered epigenetics and cancer predisposition (Borrelli et al. 2008). One of the well studied dietary factors is the amino acid deficiency that can affect the overall methylation status by altering the methyl donor availability to the methylation machinery (Waterland et al. 2006). This can often lead to the demethylation of CpG island and subsequent activation of oncogene expression in the post natal life (Doherty et al. 2000). Folate, is another important nutrient that is known to influence the promoter methylation since, it is essential for the synthesis of S-adenosylmethionine that serves as a methyl group donor (Keku et al. 2002; Piestrzeniewicz-Ulanska et al. 2004). Choline, another nutrient like folate influences

the methylation of certain gene promoters that prevents cancer in some cases while it predisposes in other instance, depending on the gene affected by DNA methylation (Zeisel et al. 2007). Alcohol consumption is also known to affect the folate metabolism. This can indirectly influence the methylome of a cell and predispose to carcinogenesis (Hamid et al. 2009).

Cancer cell is known to be associated with metabolic defects that increase the reactive oxygen species (ROS) generation which in turn alters DNA methylation status and histone modifications by oxidizing DNMTs and HMTs or they may induce genetic change through direct oxidation of nucleotide bases leading to mutation. Increased glutamine consumption in cancer cells perturbs histone acetylation and methylation due to a decrease in the NAD^+/NADH ratio creating an inhibitory environment for the activity of sirtuins and histone demethylases which ultimately liberates genes from their negative regulation (Hitchler and Domann 2009). Fiber content of diet influences the biotic population and the butyrate production in the gut. Butyrate is a well known inhibitor of HDACs. In colorectal carcinoma distinct correlation exists between dietary fiber content, HDAC activity and cancer predisposition (Corfe et al. 2009). Dietry components like diallyl disulfide from garlic and sulforaphane present in cruciferous vegetables also inhibit type I and II HDACs and prevent cancer occurrences. Curcumin and copper on ingestion induces histone hypoacetylation by inhibiting the HATs. Copper does this by triggering oxidative stress and curcumin by directly inhibiting p300 (Balasubramanyam et al. 2004; Morimoto et al. 2008). Dihydrocoumarin found in sweet clover is a well reported inhibitor of SIRT1 and disrupts heterochromatic silencing (Olaharski et al. 2005).

One other nutrient i.e., Biotin when covalently attached to histones induces gene silencing in the cellular response to DNA damage. A sudden malfunctioning of biotinylation due to biotin deficiency may hence pave way to tumorigenesis. Tryptophan and Niacin together are the precursors of nicotinamide adenine dinucleotide (NAD), that serves as a substrate for poly(ADP-ribosylation). PARYlation of histones and other DNA-binding proteins are frequently involved in DNA repair and apoptosis which when deregulated due to the deficiency of the key nutrients may lead to aberrant PARYlation and transformation (Oommen et al. 2005). The other dietary factor strongly correlated to cancer is consumption of high-fat diet. In case of rats fed with high-fat diet the ER promoter is hypomethylated and hence an increased ER expression occurs that often predisposes their offspring's to cancer (Aguilera et al. 2010).

Hazardous toxicants in the environment are also known to modulate the epigenetics of an individual. Nickel is one such toxicant that binds to heterochromatin and alters the DNA methylation in such regions apart from the decondensation of heterochromatin structures which ultimately results in epimutation leading to cancer genesis (Klein et al. 1991; Conway and Costa 1989). Apart from heretrochromatin binding in some cases people have reported that nickel can induce DNA hypermethylation and heterochromatin silencing of some tumor suppressor genes like p16. Also it can affect the lysine acetylation status of certain region based on its steric hindrance with the acetylation machinery when nickel is bound to the

Histidine residue neighboring to these lysines (Govindarajan et al. 2002; Sutherland et al. 2001). Arsenic, like nickel can also alter the DNA methylation by changing the availability of SAM which is an essential substrate for methyltransferase and also a metabolite involved in the detoxification of arsenic from the cell (Zhao et al. 1997, PNAS 94:10907–10912). Cadmium at high level acute exposures can inhibit DNMTs while the same at low level and chronic exposure can increase the activity of DNMTs (Salnikow and Zhitkovich 2008).

Some of the other contributing factors that can tamper the epigenetic homeostasis are the ageing associated hypometylation of certain gene promoters (Issa et al. 1996), pathogen induced alteration of DNA methylation and histone modifications (Maekita et al. 2006; Kalantari et al. 2004) and many such unknown factors like energy restriction (Hughes et al. 2009), alcohol consumption (Arasaradnam et al. 2008), cigarette smoking (Hobo et al. 2010) etc., have direct or indirect effects over the epigenetic deregulation especially the aberrant methylation status that are claimed to contribute towards tumor progression. Though the effects of diet and environment are well correlated to oncogenesis scientific evidences for these are only a tip of an ice berg which needs more statistically valid experiments to confirm the hypothesis.

17.4 Epigenetic Mechanisms in Cancer Manifestation

17.4.1 Epigenetics of Tumor Progression and Metastasis

The contribution of epigenetics in cancer is equally important as compared to the genetic causes of cancer. In fact it is the epigenetic status of a transformed cell that decides the course of cancer genesis by creating the second hit on the gene expression of a given tumor suppressor. Fist hit is created by the genetic mutation whereas the second hit is provided by epimutation over the second allelic wild type copy of the same gene. Since the first report came on DNA hypomethylation among the patients with colorectal cancer (Feinberg and Vogelstein 1983), the role of epigenetic mutation in various diseases pathogenesis is being widely studied. Today it is well established in the field of cancer biology that epigenetic alterations are involved in almost every step of cancer right from transformation to tumor establishment and from tumor progression to metastasis. Very much like the genetic mutation that gives rise to the property of self-sufficiency in growth signals, insensitivity towards growth inhibitory signals, ability to avert apoptosis, increased proliferation, angiogenesis to support such growth, and the ability to metastasize and invade are aided by parallel epigenetic events in such cancerous cells. As mentioned earlier this epigenetic change could be aberrant DNA methylation over the CpG islands or CpG shores which shuts down the expression of certain tumor suppressors like CDKN2A etc. or it could be a global DNA hypomethylation and activation of certain oncogenes like HRAS and KRAS (Ryan et al. 2010). It can be even a de-repression event over the repetitive sequences like transposon elements due to

altered methylation machinery or altered Piwi RNAs (Siddiqi and Matushansky 2011) which are normally involved in silencing of repetitive elements that keeps a check over chromosomal instability. Alterations in the HAT activity, like hypoacetylation over p53 are involved in diverting signals from apoptosis to survival (Sykes et al. 2006). Fusion events like MLL-CBP (Ayton and Cleary 2001), MOZ-CBP (Chan et al. 2007), MLL-p300 (Ida et al. 1997) in certain hematological malignancies are known to alter the gene expression in favor of tumor progression and angiogenesis. Inhibition of certain HDACs, like class II HDACs aid in angiogenesis and migration (Witt et al. 2009). Even an aberrant recruitment of certain HDACs may lead to certain tumor suppressor gene inactivation or an aberrant expression may cause an imbalance in the overall acetylation status within the cell (Glaser et al. 2003; Zimmermann et al. 2007).

Though numerous mutations are reported for the metastasis associated genes there are epimutations like aberrant CpG methylation, recruitment of repressive transcription factors and other chromatin modifying enzymes over these genes like cadherin genes, heparan sulfate synthesis pathway genes, tissue inhibitors of proteinases genes, laminins etc. which ultimately plays essential role in tumor cell invasion and metastasis. Moreover miRNAs are discovered to have important roles in proliferation, differentiation, apoptosis and development. Hence it is definitely not surprising to correlate miRNA and cancer progression right from oncogenesis to metastasis (He and Hannon 2004; Lu et al. 2005). The miRNA, miR-10b promotes cell migration and invasion in breast cancer by upregulating the prometastatic gene RHOC. miRNAs, miR-373 and miR-520c by suppressing CD44 aids in tumor invasion and metastasis. The oncomiR, MiR-21, inhibits multiple metastasis suppressor genes, to bring about tumor invasion and metastasis. The tumor supressor miRNAs, miR-126 regulates cell proliferation while miR-335 regulates tumor invasion. The miR-200 family miRNAs inhibits E-cadherin repressors ZEB1, ZEB2, SIP1 and transcription factor that results in epithelial- to-mesenchymal transition required for tumor metastasis (Peter et al. 2009).

17.4.2 Epigenetics and Cancer Metabolism

Metabolic reprogramming in cancer cell was first discovered by Warbug in 1956 (Warburg et al. 1956), where he found that cancer cells prefer to metabolize glucose by anaerobic glycolysis rather than oxidative phosphorylation. Numerous cell signaling events are initiated in the cancer cell when exposed to the stress created within the tumorous tissue microenvironment. The mutations which contribute towards such aberrant signaling is well studied and researchers are now trying to study the biochemical aspects of such cancer cell metabolism. One other emerging area of interest is the contribution of epigenetic alteration in initiating these aberrant metabolic events. Such precise adaptive response aids cancer cells in better survival and even gain immortality and stemness. Cancer cell metabolism can also reprogramme these cells to promote the “cancer stem cell” phenotype, by altered utilization

of metabolites. The high-energy metabolites lactate and ketone when utilized by cancer cells might induce stemness by increasing the Acetyl-CoA pool, and hence might lead to increased histone acetylation, and gene expression to create stemness in the cancer population (Martinez-Outschoorn et al. 2011). The oncogenic Myc is known to be involved in the production of Acetyl-CoA from mitochondrial metabolism by acting as a nutritional sensor during cell cycle. Acetyl-CoA produced on Myc activation is later diverted to a great extent in the acetylation of histones. Since the Myc dependant global acetylation is at the point of cell cycle entry, its deregulation is expected to predispose in cancer development (Morrish et al. 2010). Recently, it was also shown that the ATP-citrate Lyase activity is enhanced growth factor signaling that ultimately increases the nutrient metabolism. The global histone acetylation is determined by glucose availability via ACL activity regulation which leads to enhanced utilization of glucose and hence an energetically favorable state is achieved for cellular proliferation to occur. Probably, this could favor in creating an epigenetically conducive environment for the cancer cell proliferation and tumor progression (Wellen et al. 2009). In support to the same hypothesis acetyltransferases that acetylate protein substrates other than histone which posses gene regulatory function are known to have control over the metabolic genes involved in the production of metabolic fuel. At this juncture these observations encourage us to believe that one of the driving forces for tumor progression other than epigenetic modification of histone might be through regulation of certain transcription factors whose activity is dependent on the acetylation status.

Metabolic defects in most of the cancer cell leads to increased ROS production which might attack the chromatin modifying enzymes that might lose its function and cause global changes in the chromatin structures that are more prone to damage and hence, chromosomal instability ensues. Similarly due to the Warburg effect and increased glutamine consumption, the histone acetylation and methylation are affected due to the altered activity of sirtuins and histone demethylases (Hitchler and Domann 2009).

17.4.3 Epigenetics and Cancer Stem Cells

Cancer "stem/initiating cells" are considered as the seeds of a tumor tissue in most aggressive forms of cancer. These cancer stem cells possess a signature sign of increased polycomb protein group (PcG) complex members, to maintaining a poised, low basal activity, over the genes for which PcG targeting is normally key to maintain bivalent epigenetic status similar to that of the progenitor stem cell like status where both H3K4 methylation and H3K27 tri-methylation marks coexist (Ben-Porath et al. 2008; Bernstein et al. 2006). Embryonic stem cell in general maintains repression over certain sets of promoter region using PcG proteins. Surprisingly, the same sets of regions are also more likely to be methylated to permanently lock them in the stem cell state (Widschwendter et al. 2007). Similarly, *BMI*1 is a part of the polycomb group genes (PcG) that acts as a chromatin modifier

well known for its role in embryonic and stem cell self-renewal. *BMI*1 upregulation is also associated with malignant transformation and is involved in the maintenance and propogation of cancer stem cells. The DNA methylation aberrations, like global hypomethylation and promoter hypermethylation, were not rare with tumors displaying cancer stem cell properties (Widschwendter et al. 2007). The side population representing such cancer stem cell often posses DNA methylation defects and it is well correlated with increased tumorigenicity within the side population (Marquardt et al. 2010). The tumor suppressor miRNA, miR-34a negatively regulates the expression of CD44 an important marker for stemness in Prostate cancer. Thus epigenetic defects like downregulation of miR-34a leads to cancer development and manifestation of their migratory, invasive and metastatic properties (Liu et al. 2011).

Accumulation of global epimutations arises from very early alterations in the epigenetic machinery, during neoplastic evolution. Since epigenetic mechanisms are central to maintenance of stem cell identity, its disruption may give rise to a high-risk aberrant progenitor cell population. Later this population transforms upon subsequent genetic gatekeeper mutations and become cancerous (Sharma et al. 2010). On the contrary some key gate keeper epigenetic mutations are also reported to happen during stress response and turn on tumor specific marks (Baylin and Ohm 2006). Still the effects of such epimutations either in the early or late events of tumor initiation and progression has equally important roles to play as compared to the genetic mutations.

17.5 Epigenetics and Therapeutics: Promise and Challenges

Presently, there are several epigenetic drugs like inhibitors of DNMTs and HDACs are at various developmental stages while some are in clinical trials. Several laboratories around the world are busy in designing and developing inhibitors and/or modulators for targeting the enzymes that mediate the epigenetic modifications. The selection of a right target (proteins) at the right place (type of cancer) is important. Targeted delivery of these epigenetic drugs hold great promise by, decreasing there side-effect and toxicity. Further a complete understanding of the epigenetics of cancer cell is also a prerequisite for a more efficient anticancer strategy. With the ever increasing list of new epigenetic modifications on histones and its variants, nonhistone protein and there cross talk makes the task even more challenging. The role of epigenetic drugs on nonhistone proteins is also to be addressed. Combination therapies of epigenetic drugs, with other anticancer therapy provide an additional space against cancer treatment which should be exploited to its maximum benefit. Designing smart small molecule inhibitors or ligands which disrupt protein-protein interaction in various protein complexes (e.g. HAT, HDAC complexes) should also be explored while the effect of known molecules on such complexes needs to be further investigated. In the years to come we hope to understand the characteristics of cancer stem cells from different origin in the epigenetic context. The signal

dependent differentiation of the cancer stem cells could be understood more systematically by systems biology approach. Though numerous epigenetic correlations have been drawn to cancer manifestation the cause and effect is not well deciphered, which leaves a humungous task for researchers to knit the finer details in mapping the epigenetic causes of cancer.

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Chapter 18
Epigenetic Regulation of Cancer Stem Cell Gene Expression

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Abstract The concept of cancer as a stem cell disease has slowly gained ground over the last decade. A 'stem-like' state essentially necessitates that some cells in the developing tumor express the properties of remaining quiescent, self-renewing and regenerating tumors through establishment of aberrant cellular hierarchies. Alternatively, such capacities may also be reacquired through a de-differentiation process. The abnormal cellular differentiation patterns involved during either process during carcinogenesis are likely to be driven through a combination of genetic events and epigenetic regulation. The role(s) of the latter is increasingly being appreciated in acquiring the requisite genomic specificity and flexibility required for phenotypic plasticity, specifically in a context wherein genome sequences are not altered for differentiation to ensue. In this chapter, the recent advances in elucidating epigenetic mechanisms that govern the self-renewal, differentiation and regenerative potentials of cancer stem cells will be presented.

18.1 Introduction

Understanding the mechanisms that control growth and differentiation in normal cells are an essential requirement to elucidate the origin and reversibility of malignancy. In normal tissues, the generation of new cells through stem cell hierarchies is counterbalanced by apoptosis of differentiated cells, thereby maintaining a constancy of cell numbers. Once established in the normal state, temporal and spatial activation or silencing of specific genes occurs in a cell-type-specific pattern in the hierarchy to secure cell fate. Such processes are stable over several cell generations and long

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after inductive developmental signals have disappeared. Disruption of cellular homeostasis is believed to be involved in several diseases including cancer (Yamada and Watanabe 2010). Over the last few decades, understanding of such mechanisms has led to the identification of stem-like cells in tumors. The salient defining features of such cancer stem cells (CSCs) that contribute to tumor cell survival and drug resistance include the following:

(i) ***Potential to self renew*** – which involves a capability of CSCs to undergo asymmetric division in response to microenvironmental signals and maintain themselves in a state of reversible quiescence ensuring continuous regenerative potential.
(ii) ***Establishment of a cellular hierarchy*** – such a phenomenon maximizes cellular resources towards an efficient cell turnover in the normal as well as transformed tissues. The hierarchies in cancer are considered aberrant due to a differentiation block leading to accumulation of proliferating progenitors. Such a maturation arrest subsequently leads to compromised tissue functioning and could induce resistance to apoptosis during disease progression.
(iii) ***Tumor Regeneration*** – this reflects on a capability of very few CSCs to form new tumors. This presents two main clinical implications *viz.* the few migrating CSCs regenerate secondary tumors at newer sites and, post-therapy residual CSCs can lead to disease recurrence.

The last few decades have witnessed considerable focused efforts on the identification of genetic events associated with the above cellular processes. CSC research has primarily been supported by the understanding of normal stem cell function and tissue homeostasis. The availability of *in vitro* model systems including embryonic stem (ES) cells and more recently, induced pluripotent stem (iPS) cells have revealed several critical aspects of stem cell functioning that are applicable to the CSC state. Concurrently, a careful examination of diverse gene expression profiles has revealed similar patterns in several aggressive tumors and stem cells, especially of genes that contribute to the above capabilities. Stem cell features (stemness) can also be acquired by tumor cells through a phenomenon often termed as '*de*-differentiation'. This may involve aberrant expression of CSC markers *e.g.* expression of *JARID1B*, an H3K4 demethylase in malignant melanoma (Roesch et al. 2010), or through the process of epithelial-mesenchymal transition (EMT) (Kurrey et al. 2009; Mani et al. 2008). These observations suggest that the CSC phenotype is dynamically regulated, possibly through ***stochastic*** means that complements the ***hierarchical*** regenerative mechanisms identified in several tumors. Thereby both cellular models might co-exist and tumor progression could be driven by genomic changes that give rise to other CSCs and their subsequent progeny through clonal evolution (Wani et al. 2006).

In its initial phases, the focus in CSC biology was to study stem cell-specific transcription factors, including Oct4, Nanog, and Sox2 that function in regulatory complexes to determine pluripotency. Integration of these approaches with other regulatory mechanisms to define the various components involved in malignant transformation associated with a CSC phenotype are being initiated (Kashyap et al. 2009).

These increasingly suggest that the requisite genomic specificity and flexibility required to establish phenotypic plasticity and maintain "stemness" profiles involve intricate molecular networks that include epigenetic modulation of the chromatin in both – normal and transformed cell groups (Hochedlinger et al. 2005; Li and Zhao 2008; Melcer and Meshorer 2010). A defining feature of such epigenetic events that lead to heritable changes in gene expression is that they are not based on alterations in the DNA sequence (Khavari et al. 2010). In the context of cancer while a debate continues on whether these can be interpreted to be a cause or effect of the transformation process, a strong role of their contribution is definite and reveals a cross-talk with genetic mechanisms and post-translational modifications. Thereby the regulatory role of epigenetic mechanisms including DNA methylation, chromatin remodeling through histone modifications and nucleosome positioning, non-coding RNA in stem cell biology and its dysfunctional states such as cancer and developmental disorders are receiving key importance. Here, we review the recent evidences that advance our knowledge in epigenetic regulations of cancer stem cells as aberrant derivatives of adult mammalian stem cells.

18.2 Altered DNA Methylation and Cancer Stem Cell Functions

Global DNA hypo- and hypermethylation at promoter regions of genes are common features of human tumours (Ehrlich 2009; Duthie 2011). DNA methylation involves the covalent linkage of a methyl group to carbon 5 of a cytosine in cytosine-phosphate-guanine (CpG) dinucleotides. It remains the most extensively studied epigenetic modification and negatively correlates with gene expression especially in genes with promoter regions rich in CpG islands. Promoter methylation mediated gene silencing provides an alternative to mutational inactivation; the consequent loss of function through either mechanism is known to facilitate tumor initiation and progression. Increasingly several genes which would otherwise not be recognized as being contributory to normal development and cancer are being discovered as a consequence of their altered methylation profiles in tumors. This provides an enhanced understanding of genome-wide changes in the disease that correlate with aberrant cellular functions. Altered methylation is demonstrated in the acquisition of pluripotency, evasion of cell cycle check-points (through suppression of potential key tumor suppressor genes), proliferation, immortalization and genomic instability (expression of oncogenes), as well as aberrant expression of tissue-specific housekeeping and imprinted genes (De Smet et al. 1999).

Self-renewal, a defining feature of stem cells and also CSCs, is distinguished from proliferation by the capacity of one of the daughter cells to exit from the cell cycle to enter a quiescent state without losing its regeneration potential (Bapat 2007). Reprogramming of differentiated cells through transgene expression of Oct4, Sox2, Klf4 and cMyc enables them to re-acquire self-renewal and pluripotency. This is an underlying principle in the generation of induced pluripotent stem cells

(iPS) and one that has tremendous therapeutic potential (Takahashi and Yamanaka 2006). Successful reprogramming depends largely on micro-environmental factors and establishment of DNA methylation motifs in some specific CpG sites complemented by demethylation and expression of specific genes critical for acquisition of stemness (Shoae-Hassani et al. 2011). Deviations from efficient reprogramming could result in aberrant states such as transformation and an association with a differentiation block and compromised functioning of regenerated tissues (Ohi et al. 2011). Initial evidence for such a concept was the finding that abnormal promoter methylation in cancer involves several genes involved in maintenance of stem/progenitor populations in embryonic development and adult cell renewal. Moreover, the frequent establishment of these aberrant profiles occurred early during hyperplasia much before malignancy is detected (Baylin and Ohm 2006; Jones and Baylin 2007). More or recently, the introduction of two tumor suppressor genes *viz. HIC1* and *RassF1A* in a methylated (promoter) state into bone marrow-derived mesenchymal stem cells showed potential in generation of CSCs. The stem cells were identified using conventional assays for loss of anchorage dependence, increased colony formation capability, drug resistance, pluripotency, self-renewal, tumor formation, and subsequent serial xenotransplantation (Teng et al. 2011). Further, treatment of the targeted MSC with a DNA methyltransferase inhibitor reversed their tumorigenic phenotype, thereby providing a proof of concept.

Several CSCs also express the stem cell markers associated with stem cells in normal tissues. CD133, a marker associated with neural stem cells which is silenced during differentiation, sometimes presents a hemi-methylated DNA state in neuroblastoma cell lines leading to its expression; consequently it has been applied towards isolation and enrichment of putative CSCs from human tumors (Schiapparelli et al. 2010). An elaborate comparison of promoter DNA methylation patterns of cancer-related genes between human ES cells, different types of terminally differentiated tissues and cancer cell lines revealed a subset of genes methylated in ES cells and cancer (Calvanese et al. 2008). Conversely, several other genes expressed in these two cell groups were methylated in differentiated tissues, suggesting that aberrant methylation effects in cancer could also arise through defects in establishing proper methylation marks early in the cellular tissue regenerative hierarchies, rather than as an anomalous process of *de novo* hypermethylation in differentiating tissue.

At a mechanistic level, methylation patterns are established by proteins with methyltransferase activity. *De novo* methylation marks particularly during embryogenesis and cell differentiation are heritably generated by the cooperative activity of DNMT3A and DNMT3B, while maintenance of pre-existing patterns in the postnatal and adult state is mediated by DNMT1 through a preferential affinity to hemimethylated DNA during replication. Further gene silencing occurs through either preventing transcriptional activation by blocking transcription factors from accessing target-binding sites or providing binding sites for methyl-binding domain (MBD) proteins such as MeCP2, MBD1, MBD2, etc. that in turn recruit co-repressor complexes to form a repressive chromatin structure involved in transcriptional silencing (Wade 2004).

DNMT1 has been reported to be critical in regulation of the undifferentiated phenotype through suppression of differentiation in highly regenerative tissues (Sen et al. 2010). Such constitutive methylation shows dosage-dependent effects and is essential for self-renewal of multipotent and lineage-restricted progenitors as well as long-term stem cells, but may not be involved in homing, cell cycle control and suppression of apoptosis (Broske et al. 2009; Trowbridge et al. 2009). *DNMT1* is clearly aberrantly regulated in both impaired spermatogenesis and development of embryonal carcinoma (Omisanjo et al. 2007). The *de novo* methyltransferases DNMT3A and DNMT3B are also established to play a critical role in HSC self-renewal (Tadokoro et al. 2007), and in cancer (Ding et al. 2008; Van Emburgh and Robertson 2011). In mouse ES cells, DNMT3A can function as either a positive or negative transcriptional regulator (targeting the genes vitronectin and Oct3/4 respectively), that further suggests a role for ancillary molecules in decision-making (Kotini et al. 2011). Surprisingly, DNMT3L, earlier assigned to be an enzymatically-inactive DNA methyltransferase, appears to regulate the activities of DNMT3A and DNMT3B in cervical cancer cell lines (Gokul et al. 2009). The downstream effects of these changes include altered expression pattern of genes important in nuclear reprogramming, development and cell cycle, as further evinced in an altered phenotype involving increased cellular proliferation (tumor progression) and anchorage-independent growth (stemness). Other molecules such as Lsh (a regulator of repressive chromatin at retrotransposons), also play an important role in silencing of stem cell-specific genes such as *OCT4*. Specifically, Lsh is required for establishment of DNA methylation at promoters of stem cell genes during differentiation, which in part is by regulating access of DNMT3B to its genomic targets (Xi et al. 2009).

DNA methylation was earlier thought to be a relatively stable modification with demethylation occurring as a transient and passive process *e.g.* during early embryogenesis. The frequent global hypomethylation reported in cancer is sometimes associated with loss of genomic imprinting and can lead to altered cell functioning including chromosome instability, activation of transposable elements, etc. (Eden et al. 2003; Karpf and Matsui 2005). Hypomethylation of DNA repeats during tumor progression has also been described as carcinogenesis-associated demethylation since the latter may be construed to involve an exchange of C-residues for m5C residues (Ehrlich 2009). Recently, two mechanisms of DNA demethylase activity have been described – one involving the deamination of the methyl group followed by mismatch repair by activation-induced cytidine deaminase (AID) containing protein complexes, while the other mediates a direct removal and replacement of 5-methylcytosine by cytosine (Rai et al. 2008, 2010). Demethylation catalyzed by the TET (ten-eleven-translocation) proteins Tet1, Tet2 and Tet3 have been demonstrated not only to define ES maintenance and inner cell mass cell specification, but are associated with myeloid malignancies (Mohr et al. 2011). This role has been attributed to their maintenance of Nanog expression, an established marker of the undifferentiated state (Ito et al. 2010). Combined with low fidelity DNA methylation inheritance, such mechanisms could play a dynamic role in regulating cellular processes by generating variable methylation patterns and increased chances of transformation (Xie et al. 2011).

CSCs may undergo DNA demethylation during their generation, in a manner quite akin to nuclear reprogramming and generation of iPS cells. Sorted breast cancer stem cells ($CD44^{+}/CD24^{low}$ that correlate with increased mammosphere forming capabilities), showed a constitutive activation of Jak-STAT pathway through hypomethylation of several gene components (Hernandez-Vargas et al. 2011). Using undifferentiated zebrafish intestinal cells as a model system, it has been demonstrated that loss of the APC tumor suppressor gene causes upregulation of a DNA demethylase system and a concomitant hypomethylation of key intestinal cell fate genes. Mechanistically, the demethylase genes were found to be directly activated by Pou5f1 and Cebpβ, and indirectly repressed by retinoic acid that antagonizing these two genes to induce cell differentiation (Rai et al. 2010). Re-expression of certain embryonic genes e.g. Homeobox family proteins in cancer despite a lack of their expression in normal adult somatic tissues has also been attributed to aberrant demethylation patterns (Li et al. 2011; Rai et al. 2010).

Together, current findings re-emphasize the point that altered methylation status in cancer may not simply reflect promoter DNA methylation gone awry within the tissue stem or progenitor cell(s). In this altered scenario, the identification of genes that may either be hypermethylated or hypomethylated are being considered for their biomarker potential in several cancers including prostate, liver, gastric, etc. cancers (Ammerpohl et al. 2011; Ibragimova et al. 2010; Kwon et al. 2011). Recently, a SRAM (significantly repressed in association with methylation) gene signature that includes *EPCAM*, *APC*, *CDH1*, etc. has been applied to distinguish tumors of different lineages in breast cancer epithelial and mesenchymal lineages. The SRAM signature further identified the rare claudin-low and metaplastic tumors in association with mesenchymal characteristics, suggesting aberrant DNA methylation as a marker of cell lineage rather than tumor progression (Han et al. 2009; Sproul et al. 2011). Further, increasing reports on involvement of demethylases leading to a hypomethylated state that influence stem cell functions in tissue homeostasis and cancer reflect on more intricate methylation dynamics that realized earlier. The emerging point of view is of considerable interest in epigenetics and cancer research at present.

18.3 Histone Modifications and Cancer Stem Cell Functions

Post-translational chemical modifications of amino acid residues on histone ‘tails’ of the nucleosome are established and removed by a network of macromolecular complexes composed of molecules with recognition domains and enzymatic activity including histone acetyltransferases (HATs) and histone methyltransferases (HMTs) that add acetyl and methyl groups respectively, while histone deacetylases (HDACs) and histone demethylases (HDMs) remove these groups. Such complexes also interact with each other and establish cross-talk with other DNA regulatory mechanisms to tightly link the chromatin state and transcription. The specific histone residue modified and the type, location and degree of modification(s) enriched at promoter

regions of genes can define the state of expression. This is the crux of the 'histone code'. A general derivation based on their spatial effects on chromatin architecture is that acetylation correlates with transcriptional activation, whereas methylation can lead to either activation or repression. For example, histone acetylation is sufficient to mediate the activation of *NESTIN* transcription in the absence of DNA demethylation (Han et al. 2009). More specific associations of trimethylated lysine 4 on histone H3 (H3K4me3) enrichment at transcriptionally active gene promoters, and trimethylated lysine 9 on histone H3 (H3K9me3) and trimethylated lysine 27 on histone H3 (H3K27me3) with silent gene promoters have also been established (Portela and Esteller 2010; Weishaupt et al. 2010). Stem cells are known to exhibit an association of active chromatin states (defined by H3K4me3) around pluripotency genes and silencing of cell fate- and differentiation- specific genes mediated by H3K27me3 (Boyer et al. 2006; Chi and Bernstein 2009; Mikkelsen et al. 2008). Under-representation of repressive histone marks could be indicative of epigenetic plasticity in stem, young and tumor cells, while committed and senescent (old) cells often display increased levels of these more stable repressive modifications (Kubicek et al. 2006).

The co-existence of two histone modifications with opposing effects at the same promoter region describes 'bivalent domains' that have established their own connotation with biological functions (Chi and Bernstein 2009; Mikkelsen et al. 2008). Genome-wide profiling of histone marks in undifferentiated stem cells identified active H3K4me3 and repressive H3K27me3 marks to be enriched at promoters of developmentally important genes (Bernstein et al. 2006). Such bivalency maintains transcriptional quiescence or poise yet permits the flexibility essential for divergence along an alternate cell fate(s). Subsequently such marks are selectively lost during differentiation to define specific lineage commitment and functionality. Establishment of the H3K4me3-H3K27me3 mark involves activation of two opposing regulator complexes *viz.* trithorax group that catalyzes the H3K4me3 mark and the polycomb group that establishes H3K27me3 marks (Lund and van Lohuizen 2004; Orlando 2003; Ringrose and Paro 2007; Valk-Lingbeek et al. 2004).

The selective transition from bivalent to repressive histone modifications in cell cycle regulatory genes, yet retention of bivalent promoters in developmental genes has been identified to be a key feature in transformation (Huang and Esteller 2010). Cultured mammary epithelial cells routinely enter into senescence and growth arrest after a few passages; however, rare progenitor cell(s) may proliferate and develop into neoplastic clone(s) (Hinshelwood and Clark 2008). Such aberrant progenitors while retaining bivalent features to regulate differentiation-associated genes (leading to lack of lineage commitment and/or maturation arrest), enrich the H3K4me3 mark to activate stemness-associated genes, and the H3K27me3 mark to mediate silencing of tumor suppressor genes. Thus, multipotent renal progenitor cells residing within the nephrogenic mesenchyme, which are under tight control during renal development, undergo sequential epigenetic alterations that mediate silencing of the nephric-progenitor genes such as *SIX2*, leading to tumor initiation and progression (Metsuyanim et al. 2008). In normal stem cells, H3K9 acetylation and demethylation regulates self-renewal through canonical signaling, cell cycle and cytokine related pathways (Tan et al. 2008). In cancer, H3K9me2 / H3K9me3 often

serve to enhance H3K27me3-mediated silencing to drive effective transformation (Bloushtain-Qimron et al. 2008; Cheng et al. 2008; Hsu et al. 2009).

Aberrant H3K4me3 marks leading to compromised cell fate decisions and cancer correlates with dysfunctional SET1/MLL histone methyltransferase complex components such as Dpy-30 (Jiang et al. 2011) or complex recruiting factors such as Pygo2 (Duan et al. 2005; Gu et al. 2009). The Gfi1 transcriptional regulator oncoprotein requires histone lysine methyltransferase G9a, histone deacetylase 1 (HDAC1) and H3K9me2 in repressing the cell cycle regulator p21Cip/WAF1 (Duan et al. 2005). While histone demethylases have also been shown to play a key role in eukaryotic gene transcription; the specific molecular networks coordinated by them towards regulation of specific target genes and biological processes remains to be elucidated (Di Stefano et al. 2011).

Histone-modifier genes usually have tissue-type specific patterns of expression in cancer, and the landscape varies when solid tumors or hematological malignancies are compared (Ozdag et al. 2006). The contributions of Polycomb group proteins (PRCs) remain the best studied mechanism associated with histone modifications (Margueron and Reinberg 2011) since its functional versatility contributes significantly to the complexity of regulatory pathways in cancer (Kanno et al. 2008). The PRC2 complex (EZH2, SUZ12, EEX) initiates gene silencing by methylating H3K27, while PRC1 (Bmi1 being a prototype component) maintains gene silencing through mono-ubiquitination of histone H2A lysine 119. Expression of PRC2 components is regulated by the retinoblastoma protein (pRB)–E2F transcription factors, and are consequently overexpressed in various cancers such as melanoma, lymphoma, and breast and prostate cancer (Bracken et al. 2003; Karanikolas et al. 2009; Li et al. 2009; Margueron et al. 2008). On the other hand, depletion of EZH2 leads to enhanced activation of the Ink4/Arf locus that regulates expression of the cell cycle checkpoints p16Ink4a, p19Arf and p15Ink4b (Bracken et al. 2007; Ezhkova et al. 2009). PRC2 is also required for the acquisition of pluripotency, as $Eed^{-/-}$ and $Suz12^{-/-}$ ES cells fail to induce the reprogramming of B cells (Pereira et al. 2010). EZH2 is identified to play a crucial role in neural stem cell self-renewal and proper lineage commitment (Sher et al. 2008). The epithelial cell adhesion molecule (EpCAM) expressed in several progenitor cell populations regulates the reprogramming genes c-MYC, OCT4, NANOG, SOX2, and KLF4 and is thereby critical in maintenance of the undifferentiated state. EpCAM is silenced in some tumors through establishment of a H3K27me3 mark at its promoter by SUZ12 and JMJD3, leading to a perturbed cellular hierarchy (Lu et al. 2010). A novel PcG protein PCL2 has been identified in association with enhanced ES self-renewal, delayed differentiation and altered patterns of histone methylation (Walker et al. 2010). Depletion of PCL2 leads to decreased H3K27me3 and increased expression of the pluripotency transcription factors Tbx3, Klf4, Foxd3, that subsequently activate expression of Oct4, Nanog and Sox2 through a feed-forward gene regulatory circuit, altering the core pluripotency network and driving cell fate decisions towards self-renewal (Walker et al. 2011). Another PcG component L3MBTL is located at an imprinted locus at 20q, and is implied in the pathogenesis of myeloid malignancies associated with 20q deletions (Li et al. 2004).

Co-operation and co-ordination between PRC1 and PRC2 components is also essential for normal stem cell functioning. The p16 locus is reported silenced through association with H3K27me3, BMI1, RING2, and SUZ12. This is mediated by the pRB proteins that recruit PRC2 to trimethylate p16, priming the BMI1-containing PRC1L ubiquitin ligase complex to silence p16 (Kotake et al. 2007). While the role of Bmi1 in stem/progenitor cells self-renewal is well established, in hepatic stem cells Ezh2 additionally plays an essential role in maintenance of not only their proliferative and self-renewal functions, but also full execution of a differentiation program (Aoki et al. 2010). Importantly, Ezh2 depletion (but not Bmi1) promoted up-regulation of several transcriptional regulators leading to differentiation and terminal maturation of hepatocytes; thereby emphasizing a functional non-redundancy of the PRC2 and PRC2 mediated chromatin regulatory mechanisms.

Taken together, the above data emphatically supports the hypothesis that deregulation of the 'histone code' in cells with pluripotent potential may alter defining properties of stem cells, self-renewal and differentiation potential, leading to cancer initiation and progression (Biancotto et al. 2010; Shukla et al. 2008), and this could happen through *de novo* "writing", "erasing" or "misinterpretation" of histone marks by activities of the regulatory complexes (Yoshimura 2009).

18.4 Cross-Talk Between DNA and Histone Methylation

Chromatin thus is dynamically active in regulating transcriptional processes through post-synthetic modifications of DNA and histones. At a higher level, DNA and histone associations also cooperate to impart stability to this regulation with aberrant CpG hypermethylation events in cancer being more frequently associated with the promoters of those genes with enriched PRC occupancy. Such cooperative events increase with age and may contribute to carcinogenesis by irreversibly silencing genes that are suppressed in stem cells, as demonstrated through the identification of an age-PRC target methylation signature present in preneoplastic conditions that drive transformation associated gene expression changes (Teschendorff et al. 2010).

The fact that stem cell associated PRC targets appear to have an increased propensity for cancer-specific promoter DNA hypermethylation than non-targets, supports a stem cell origin of cancer in which reversible gene repression is replaced by permanent silencing, locking the cell into a perpetual state of self-renewal and thereby predisposing it to subsequent malignant transformation (Widschwendter et al. 2007). During renal tumorigenesis in stem cell-like Wilms' tumor xenografts, the PcG components EZH2, BMI1, EED and SUZ12 showed cooperative upregulation along with nephric-progenitor genes WT1, PAX2 and SALL1 although SIX2 was downregulated through promoter hypermethylation leading to loss of renal differentiation. This links polycomb activation and promoter methylation in renal progenitor populations during the initiation and progression of renal cancer (Metsuyanim et al. 2008). Such polycomb-premarking has been also identified in colorectal cancer (Rada-Iglesias et al. 2009).

It is also indicated that the key role of promoter DNA hypermethylation in cancer cells is to tightly repress the transcription of specific genes beyond the repression achieved in the bivalent chromatin state. Higher order repression may further be established by looping in the 3′ region of such genes that further encompass multiple, abnormally hypermethylated CpG islands surrounding the entire gene resulting in no basal transcription. This is also evinced by very low levels of Pol2 polymerase at the transcription start site(TSS) and enrichment of the methyl-cytosine binding protein, MBD2 at the CpG islands around the TSS which are classically assessed for DNA methylation (Akiyama et al. 2003; Tiwari et al. 2008). Such stable repression is difficult to revert by simple targeting through demethylation. For example, in breast and colon cancer chromatin immunoprecipation (ChIP)-chip tiling arrays (McGarvey et al. 2006, 2008) have revealed that hypermethylated gene promoters are also enriched with a series of repressive chromatin marks including H3K27me3. While promoter DNA demethylation of these genes and loss of MBD2 either by treatment with the demethylating agent, 5-deoxy-azacytidine (DAC) or by genetic knockout of DNA methyltransferases (*DNMT1* and *DNMT3b*) could be induced (Rhee et al. 2002), repressive H3K27me3 remained and with a concurrent repositioning of the active H3K4me2 mark, led to the formation of a bivalent chromatin state with continuing low level gene expression.

Formation of the above "repressive hubs" draws attention to the dissimilarities between the epigenetic regulatory mechanisms higher order chromatin conformation in CSCs and for the same gene(s) in stem/progenitor cells. In the normal state, activation is induced through dissolution of loops and consequent signal transduction induced cell differentiation. In cancer cells however such genes although appear to be 'poised' for differentiation through the presence of bivalent histone marks, are often further and heritably repressed through additional histone marks and higher order chromatin compaction mechanisms including DNA methylation that effectively renders the gene inaccessible for the transcription required to facilitate conversion to a differentiated state. This effectively establishes the maturation arrested state characteristic of a CSC hierarchy.

18.5 Conclusion and Future Perspectives

The deluge of new discoveries relating to epigenetics in the last few years including establishment of comprehensive DNA methylomes of normal and aberrant states, identification of non-CpG methylation, the definition of CpG island shores, description of new histone modifications and histone variants and their roles, reports of mutations in the epigenetic machinery, the flurry of miRNA-ncRNA studies and the cross-talk between all these mechanisms that would reflect on higher order chromatin regulatory mechanisms, together highlight the recognition of epigenetic mechanisms in homeostatic mechanisms in cells. The acquisition and maintenance of cell fate or "identity" by strict coordination between genetic and epigenetic programs are essential for growth and development. Cancer cells possess traits reminiscent of

those ascribed to normal stem cells. Histologically poorly differentiated tumors show preferential overexpression of genes normally enriched in ES cells combined with repression of polycomb-regulated genes and overexpression of activation targets of Nanog, OCT4, SOX2 and c-Myc. On similar lines, an epigenetic stem cell signature may be defined with reference to DNA promoter hypermethylation of polycomb group targets and bivalent chromatin that is described in human tumors. This suggests that understanding the mechanisms by which pluripotency transcription factors, complexes imparting/removing acetylation and methylation modifications, polycomb repressive complexes, histone modifications and higher order chromatin compactions maintain the balance between self-renewal, cellular proliferation and differentiation in both normal and aberrant states is important. Such links are quite compelling for continuing research towards validation or refuting various hypotheses that could enhance our understanding of the biology of cancer initiation.

However, several key questions remain:

- ***Are specific epigenetic modifications a cause or a consequence of aberrant cellular differentiation?***
- ***What mechanisms convey sequence specificity to the complexes involved?***
- ***How can causative (driver) epigenetic changes be distinguished from bystander events?***
- ***Can the threshold for accumulation and complementation of genetic and epigenetic changes in a pathological condition be defined?***
- ***What restricts formation of "repressive hubs" around developmental genes in normal stem cells?***

The establishment of meaningful stem cell models for addressing these questions will be instrumental towards understanding the dynamics of epigenetics in stem cells, development, cancer and aging. Such a research focus will be key in our progress in tackling tumor formation and disease progression.

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Chapter 19
Role of Epigenetic Mechanisms in the Vascular Complications of Diabetes

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Abstract Diabetes and metabolic disorders are leading causes of micro- and macrovascular complications. Furthermore, efforts to treat these complications are hampered by metabolic memory, a phenomenon in which prior exposure to hyperglycemia predisposes diabetic patients to the continued development of vascular diseases despite subsequent glycemic control. Persistently increased levels of oxidant stress and inflammatory genes are key features of these pathologies. Biochemical and molecular studies showed that hyperglycemia induced activation of NF-κB, signaling and actions of advanced glycation end products and other inflammatory mediators play key roles in the expression of pathological genes. In addition, epigenetic mechanisms such as posttranslational modification of histones and DNA methylation also play central roles in gene regulation by affecting chromatin structure and function. Recent studies have suggested that dysregulation of such epigenetic mechanisms may be involved in metabolic memory leading to persistent changes in the expression of genes associated with diabetic vascular complications. Further exploration of these mechanisms by also taking advantages of recent advances in high throughput epigenomics technologies will greatly increase our understanding of epigenetic variations in diabetes and its complications. This in turn can lead to the development of novel new therapies.

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19.1 Introduction

Diabetes is the leading cause of macro- and microvascular complications such as atherosclerosis, hypertension, nephropathy, retinopathy and neuropathy (Beckman et al. 2002; He and King 2004; Sharma and Ziyadeh 1995). These vascular complications affect all major organs leading to heart failure, kidney failure, blindness and limb amputation and can significantly enhance mortality rates in patients with diabetes relative to the normal population. Current projections predict a significant surge in obesity, diabetes and related metabolic disorders worldwide and in particular among the younger population, likely due to changes in lifestyle. This can greatly increase the economic burden associated with diabetes and its complications. Intense efforts are therefore needed to find more effective therapeutic approaches to curb the progression of diabetic complications. Diabetes increases the blood glucose levels(hyperglycemia) as a result of lack of insulin production (type 1 diabetes, T1D) or increased insulin resistance(type 2 diabetes, T2D), with the latter often being associated with hyperlipidemia. Hyperglycemia has been implicated in chronic inflammation and increased oxidant stress, the major risk factors for the development of vascular complications (Giacco and Brownlee 2010; Sheetz and King 2002; Devaraj et al. 2010). Clinical trials have demonstrated that strict glycemic control is critical to reduce the incidence of diabetic complications. They also showed that diabetic patients are at continued risk for increased vascular complications even long after achieving normal blood glucose levels, suggesting a 'metabolic memory' or 'legacy' effect of prior hyperglycemic exposure (Writing Team DCCT/EDIC Research Group 2002; Colagiuri et al. 2002). These clinical studies were further supported by experimental evidence showing that vascular cells exposed to diabetic milieu retain their pro-inflammatory diabetic phenotype for extended periods even after glucose normalization (Ihnat et al. 2007a; Villeneuve and Natarajan 2010; Pirola et al. 2010). Metabolic memory is a major challenge in the prevention of diabetic vascular complications and it is imperative to examine the molecular mechanisms involved and develop novel therapies.

Transcription regulation plays a central role in the expression of inflammatory and other pathologic genes. In general, the recruitment of transcription factors (TFs) to the cis-elements located in the promoters and enhancers plays a key role in gene regulation. However, it is now clear that epigenetic mechanisms in chromatin, i.e., changes that occur without alterations in the DNA sequence, also play important roles in gene transcription. These mechanisms control chromatin access to transcription regulators in mammalian cells and dictate active or repressed states of genes (Li et al. 2007; Murr 2010; Kouzarides 2007; Bannister and Kouzarides 2011). Furthermore, environmental factors and nutrients can affect epigenetic states and regulate the expression of genes associated with various diseases including cancer and diabetes (Sharma et al. 2010; Liu et al. 2008; Ling and Groop 2009). Recent studies have also implicated epigenetic mechanisms in the phenomenon of metabolic memory. An increased understanding of these changes in chromatin events can yield critical new information about the metabolic memory of vascular

complications and aberrant expression of inflammatory genes under diabetic conditions that can lead to the identification of novel new therapeutic targets. The current review discusses the role of epigenetics in diabetic vascular complications and some of the recent developments in this area.

19.2 Inflammatory Gene Expression and Vascular Complications

Diabetes is associated with significantly accelerated rates of vascular diseases like atherosclerosis which is multi-cellular in nature involving interactions between endothelial cells (EC), vascular smooth muscle cells (VSMC) and monocytes in the vessel wall (Ross 1999). Endothelial dysfunction induced by oxidant stress, oxidized lipids and other inflammatory mediators increases monocyte adhesion to EC, which then migrate into subendothelial space where interaction with VSMC promotes their differentiation into macrophages. The uptake of oxidized lipids by macrophages leads to foam cell formation. VSMC proliferation and migration to the sites of lesion also contribute to the formation of the atherosclerotic plaque. In advanced stages of the disease plaque rupture releases pro-inflammatory and pro-thrombotic mediators resulting in stroke (Ross 1999). EC dysfunction and macrophage infiltration also play key roles in diabetic nephropathy and retinopathy (King 2008; Giacco and Brownlee 2010; Wang and Harris 2011). All cell types involved in vascular complications produce inflammatory cytokines and chemokines, and regulate the functions of each other through autocrine and paracrine actions. Pro-inflammatory chemokines such as monocyte chemoattractant protein-1(MCP-1), cytokines including interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF-α), and growth factors such as Angiotensin II(Ang II) and macrophage colony stimulating factor (M-CSF) play key roles in the initiation and progression of vascular complications (Charo and Taubman 2004; Libby et al. 2002; Weiss et al. 2001). Several studies have demonstrated that diabetes exacerbates the production of inflammatory mediators in the vessel wall leading to further acceleration of vascular dysfunction. Enhanced activity of the pro-inflammatory TF NF-κB plays a key role in the increased inflammatory gene expression in VSMC, EC and monocytes under diabetic conditions. These cells exhibit enhanced oxidant stress and inflammatory genes such as TNF-α, MCP-1, Fractalkine (CX3CL1), IL-6, M-CSF and arachidonic acid metabolizing enzymes such as cyclooxygenese-2 (COX-2) and lipoxygenases, and the receptor for advanced glycation end products (RAGE) under diabetic conditions (Devaraj et al. 2006; De Martin et al. 2000; Barnes and Karin 1997; Brownlee 2001; Natarajan and Nadler 2004; Shanmugam et al. 2003a, b; Guha et al. 2000; Li et al. 2006; Reddy et al. 2009; Hatley et al. 2003; Min et al. 2010; Meng et al. 2010; Villeneuve et al. 2008; Yan et al. 2009). While the transcription of inflammatory genes is most often regulated by NF-κB TF (Barnes and Karin 1997; Glass and Witztum 2001), other TFs such as CREB and STATs have also been implicated (Reddy et al. 2006, 2009; Sahar et al. 2005, 2007; Chava et al. 2009). Biochemical

studies have also established the role of multiple upstream signal transduction mechanisms involving increased oxidant stress, polyol pathway, AGEs and RAGE, Protein kinase C (PKC), AT1R, oxidized lipids, tyrosine kinases and mitogen activated protein kinases (MAPKs) that can lead to the activation of TFs regulating genes involved in diabetic complications (Brownlee 2005; Natarajan and Nadler 2004; Yan et al. 2008; Marrero et al. 2005; Reddy et al. 2006). However, therapies based on these mechanisms have not been fully adequate to effectively prevent the progression of various diabetic vascular complications suggesting the need to explore additional mediators and drug targets.

19.3 Metabolic Memory of Diabetic Vascular Complications

Landmark clinical trials such as the Diabetes Control and Complications Trial (DCCT) with T1D patients showed that intensive glycemic control is critical for the prevention or reduction of long term vascular complications. In the follow up Epidemiology of Diabetes Interventions and Complications study (EDIC), the DCCT enrollees were subsequently monitored after placing both the conventional and intensive treatment groups on the same intensive glycemic control. Results from EDIC showed that patients previously receiving conventional therapy during DCCT continued to develop micro-and macrovascular complications at a much greater rate relative to those in the intensive treatment group throughout (Writing Team DCCT/EDIC Research Group 2002; Nathan et al. 2005). In other clinical trials involving T2D patients, levels of hyperglycemia at the time of diagnosis correlated with risk for developing complications in T2D patients (Colagiuri et al. 2002). Furthermore, fluctuations in post-prandial glycemic levels have also been implicated in the increased risk for vascular complications (Ceriello et al. 2008). Overall, these clinical studies have suggested that the persisting effects of prior exposure to hyperglycemia termed ‘metabolic memory’ or “legacy effect” can have long lasting deleterious effects in diabetes patients.

Studies using cell culture and animal models further established the role of metabolic memory in vascular complications. These included demonstration of increased oxidative stress, fibrotic and inflammatory gene expression in high glucose (HG) treated EC even several days after return to normal glucose (Ihnat et al. 2007b; Roy et al. 1990; El-Osta et al. 2008). VSMC derived from leptin receptor deficient *db/db* mice, a model of T2D, exhibited enhanced pro-inflammatory gene expression, monocyte binding and migration even after culturing in vitro for a few passages compared with non-diabetic *db/+* controls (Li et al. 2006; Villeneuve et al. 2008). VSMC from *db/db* mice also showed persistently increased activation of signal transduction pathways such as oxidant stress, tyrosine kinase, MAPK and downstream pro-inflammatory TFs NF-κB and CREB (Li et al. 2006). EC and macrophages from *db/db* mice also exhibited enhanced inflammatory genes and activation of NF-κB, suggesting that diabetes induces a pre-activated phenotype in target cells involved in vascular complications (Hatley et al. 2003; Li et al. 2006; Wen et al. 2006). In retinal EC, HG inhibited the expression of the antioxidant gene superoxide

dismutase (*sod2*) and this persisted even 4 days after reversal to normal glucose (Zhong and Kowluru 2011). In streptozotocin injected dogs with T1D, retinal complications persisted even after achieving normoglycemia with islet transplantation (Engerman and Kern 1987). Furthermore, in T1D rats, poor glycemic control for short periods followed by intensive glycemic control prevented the development of retinopathy. However, intensive glycemic control could not prevent retinopathy and related biochemical parameters in animals that had poor glycemic control for much longer durations (Chan et al. 2010; Kowluru 2003). Together these studies demonstrated the role of metabolic memory in diabetic complications and recent studies now suggest that epigenetic mechanisms may be involved in these events.

19.4 Epigenetic Mechanisms of Gene Regulation in Chromatin: DNA Methylation and Histone Post Translational Modifications (PTMs)

In mammalian cells, chromosomal DNA is tightly packaged into chromatin by histone proteins along with other chromatin assembly factors. Chromatin is a highly organized structure consisting of numerous nucleosome particles. Each nucleosome is composed of about 147 bp DNA wrapped around an octamer histone protein complex made up of dimers of core histone proteins H2A, H2B, H3 and H4 (Li et al. 2007; Workman and Kingston 1998). The histone proteins were initially proposed to be passive components supporting chromatin structure. However, it is now clear that they actively participate in transcriptional regulation. Epigenetic mechanisms regulate dynamic switching of chromatin between active (euchromatin) and inactive (heterochromatin) states that determines the transcription status of target genes and the biological outcomes (Li et al. 2007; Kouzarides 2007; Murr 2010). These mechanisms include DNA cytosine methylation, covalent post translational modifications (PTMs) of nucleosomal histones, small non-coding RNAs or microRNAs (miRNAs) and large intergenic noncoding RNAs (lincRNAs) (Murr 2010). While epigenetics usually refers to heritable changes, including those conferred mitotically or meiotically, that occur without changes in DNA sequence, more recently the definition has been modified to include the structural adaptation of chromosomal regions (Bird 2007). Thus, both DNA methylation and histone PTMs can work together to control epigenetic transmission (Bird 2007; Berger et al. 2009; Portela and Esteller 2010). Epigenetic mechanisms genomewide are now termed as the 'Epigenome' and major efforts are underway to understand how alterations in epigenome status can modulate diverse patho-physiolological conditions (Bernstein et al. 2007; Maunakea et al. 2010), especially since epigenetic changes can be induced by environmental factors.

DNA methylation which occurs at cytosine residues in CpG dinucleotides is one of the most stable epigenetic marks (Miranda and Jones 2007). CpGs can occur in groups called 'CpG islands' near promoters and methylation of promoter CpG islands often leads to gene repression. DNA methylation is regulated by DNA methyl transferases that mediate the transfer of methyl groups from S-adenosyl methionine (SAM). Recent studies have also implicated a role for DNA de-methylation in

cellular processes but the identity of the enzymes that mediate DNA demethylation is not fully clear (Wu and Zhang 2010). Abnormal DNA methylation or demethylation at the promoters of oncogenes and tumor suppressors is a major mechanism involved in the development of cancer (Miranda and Jones 2007; Sharma et al. 2010). However, only limited information is available on the role of DNA methylation in diabetes, metabolism and vascular complications. Some of these include the demonstration of DNA methylation in *Agouti* gene expression (Morgan et al. 1999), DNA hypomethylation in aortic tissues from atherosclerosis animal models (Turunen et al. 2009) and differential DNA methylation in monocytes of T1D patients with diabetic nephropathy compared with patients who did not develop nephropathy (Bell et al. 2010). Further information on DNA methylation related to vascular complications can be obtained from recent reviews (Dong et al. 2002; Turunen et al. 2009; Reddy and Natarajan 2011).

Histone PTMs can play major roles in the regulation of gene expression by modifying chromatin structure and by providing anchoring sites for co-activators, co-repressors and other chromatin proteins. Histone PTMs in association with other epigenetic mechanisms such as DNA methylation can modulate the euchromatin (accessible) or heterochromatin (inaccessible) status of chromatin (Murr 2010; Kouzarides 2007; Bannister and Kouzarides 2011). Several histone PTMs have been identified including phosphorylation (serine and threonine), acetylation (lysine), methylation (lysine and arginine), ubiquitination (lysine) and sumoylation (lysine) (Kouzarides 2007). Histone PTMs usually act in concert with each other to form a 'histone code' that dictates the transcriptional states of genes leading to biological phenotypes (Jenuwein and Allis 2001). The function of histone acetylation and methylation has been widely studied in the pathogenesis of cancer (Portela and Esteller 2010). Recent studies have examined their role in diabetes and vascular complications.

19.5 Histone Lysine Acetylation and Methylation in Gene Regulation

In general histone lysine acetylation (HKAc) promotes formation of open chromatin and is associated with active promoters. Acetylation of amino-terminal residues in histone tails neutralizes the positive charge weakening DNA-histone and nucleosome-nucleosome interactions (Kouzarides 2007; Roth et al. 2001). Furthermore, acetylated lysines can provide binding sites for chromatin remodeling proteins. These events can relax chromatin structure and make it more accessible to transcription factors and other regulators to promote gene transcription. HKAc is mediated by histone acetyl transferases (HATs). Several HATs including CBP, p300, pCAF, Tip60, SRC-1, SRC-2 and SRC-3 also act as co-activators. HATs can acetylate multiple lysine residues of histone and non-histone proteins (Roth et al. 2001; Kouzarides 2007). Genomewide studies using chromatin immunoprecipitation linked to Next Generation DNA Sequencing (ChIP-Seq) revealed differential regulation of specific promoters by individual HATs (Ramos et al. 2010).

Interestingly, ChIP-Seq and ChIP coupled with microarray analysis (ChIP-on-chip) studies also showed that p300 is recruited to enhancers and its location along with other histone PTMs such as H3K4me1 could be used to predict enhancers genome-wide in mammalian cells (Visel et al. 2009; Hon et al. 2009; Jin et al. 2011).

Histone acetylation is removed by histone deacetylases (HDACs), which are sub-divided into four groups (Class I, Class II, Class III and clas IV) depending on structure, function and mode of action. Class III proteins also known as Sirtuins are unique in that they require NAD^+ as co-factor, and respond to changes in metabolic status (Kouzarides 2007; Yang and Seto 2007). Removal of acetyl groups by HDACs restores the positive charge of histone lysines and promotes chromatin condensation, leading to reduced TF accessibility and inhibition of transcription. The functions of HDACs are quite complex mainly due to their low substrate specificity. However, studies in knockout mice showed they could be involved in specific function such as embryonic stem cell differentiation and EC proliferation (Dovey et al. 2010; Margariti et al. 2010). Overall, histone lysine acetylation can play significant roles in gene regulation related to biological and pathophysiological states.

Histone lysine methylation (HKme) on the other hand is associated with both active and inactive promoters depending on the specific lysine residue methylated. Furthermore, these lysines can be mono, di or tri methylated adding another level of complexity (Shilatifard 2006; Kouzarides 2007; Bannister and Kouzarides 2011). Histone H3 lysine 9 methylation (H3K9me2/3), H3K27me2/3, and H4K20me3 are generally associated with gene repression, while histone H3K4me1/2/3 and H3K36me3 are usually associated with active genes and gene bodies. Histone methyl transferases (HMTs) catalyze HKme while histone demethylases (HDMs) mediate removal of methyl groups. HMTs and HDMs are quite specific, e.g., SUV39H1 HMT mediates H3K9me2 and H3K9me3, and the MLL family members mediate H3K4me1-me3. Conversely, lysine specific demethylase 1(LSD1) removes H3K4 -me1 and -me2, and Jhdm2a demethylates H3K9me2 (Kouzarides 2007; Shi and Whetstine 2007; Trojer and Reinberg 2006). Most HMTs contain the SET domain which mediates lysin methyltransferase activity (Kouzarides 2007). The discovery of several HDMs demonstrated that HKme is indeed reversible and established the dynamic regulation of HKme in gene expression. Lysine methylation marks are recognized by chromo, tudor, MBT and nonrelated PHD domain containing proteins, which regulate the function of HKme (Kouzarides 2007).

Increasing evidence supports the critical function of HKme in diverse pathophysiological conditions including cancer (Bhaumik et al. 2007; Portela and Esteller 2010) and recently in diabetes and its vascular complications (Villeneuve and Natarajan 2010; Pirola et al. 2010; Giacco and Brownlee 2010; Reddy and Natarajan 2011). Because HKme is relatively more stable than other modifications, it is likely to be involved in transcriptional memory. Interestingly, studies showed that the polycomb group of proteins that mediate H3K27me3 remained bound to chromatin during DNA replication implicating a potential epigenetic role for such histone marks in the maintenance of transcription memory (Bantignies and Cavalli 2006; Francis et al. 2009). Thus, HKme could be a mediator of metabolic memory resulting from exposure to environment and dietary factors and diabetic stimuli such as HG, AGEs and oxidized lipids.

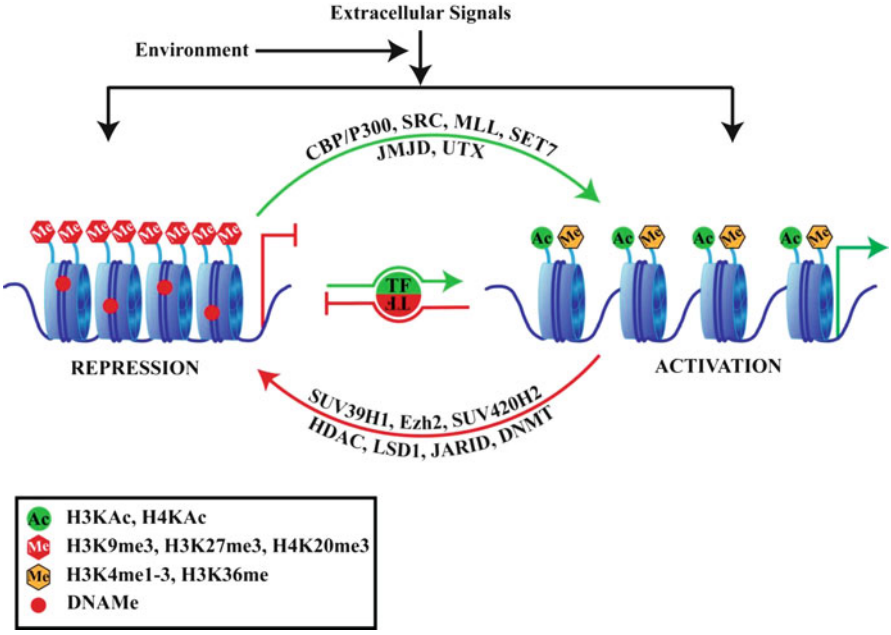

Fig. 19.1 Role of epigenetic mechanisms in gene transcription. Epigenetic mechanisms play central roles in gene regulation mediated by transcription factors (TF) by maintaining active (euchromatin) or repressed (heterochromatin) states of chromatin. These include DNA methylation (DNAMe) and histone post translational modifications (PTMs). In the repressed state, chromatin at repressed genes can be enriched with DNAMe [mediated by DNA methyl transferases (DNMTs)] and repressive histone PTMs such H3K9 methylation (H3K9me), H3K27me and H4K20me mediated by histone methyltransferases (HMTs) SUV39H1, Ezh2 and SUV420H2 respectively. In active states, chromatin is enriched by H3K acetylation (H3KAc) and H4KAc, and H3K4me and H3K36me. Histone acetyltransferases (HAT) such as CBP/p300 and SRC-1 mediate acetylation, while H3K4me is catalyzed by HMTs such as SET7 and MLL family members. Actions of HATs are opposed by histone deacetylases (HDAC). Actions of HMTs are countered by histone demethylases (HDM) such as LSD1 and JARID, which erase H3K4me marks and the JMJD family members, which erase H3K9me and H3K27me marks. This results in gene repression or activation depending on the modification that has been erased. Environmental factors and extracellular signals can affect epigenetic states to modulate the expression of genes associated with various diseases including cancer and diabetes. Ac-lysine acetylation; Me: lysine methylation

Arginine methylation of histones (HRme) can be associated with gene activation or repression. Arginine can be mono or dimethylated, and the dimethylation can be asymmetric or symmetric depending on the methyltransferase involved (Kouzarides 2007). At least 9 protein arginine methyltransferases (PRMTs) have been identified including coactivator-associated arginine methyltransferase 1 (CARM1). HRme is removed by arginine demethylases such as JMJD6. HRme might play key role in inflammation, cell proliferation and differentiation (Wysocka et al. 2006; Miao et al. 2006).

Role of epigenetic mechanisms including histone PTMs and DNA methylation in gene transcription and interaction with environment is summarized in Fig. 19.1.

Recent advances in next generation sequencing (NGS) have significantly enhanced our technical capabilities to analyze genomewide changes in histone PTMs and DNA methylation (Metzker 2010; Hawkins et al. 2010; Maunakea et al. 2010). This area is poised to make tremendous progress towards understanding the function of epigenetic mechanisms under normal and disease states.

19.6 Histone Modifications in Diabetes

Histone modifications were shown to play key role in pancreatic islet specific expression of insulin and related genes in response to changing glucose levels. Under HG conditions, the islet specific transcription factor Pdx1 was shown to recruit a co-activator HAT p300 and a H3K4 methyltransferase SET7/9 at the insulin promoter. This was associated with increased promoter levels of the activation marks H3K9/14Ac and H3K4Me and insulin expression. Conversely, under low glucose conditions, Pdx1could recruit HDAC1 and HDAC2 to the insulin promoter, leading to the inhibition of insulin expression. Interestingly, Pdx1 also induced SET7/9 expression, which in turn increased the expression of genes involved in glucose induced insulin secretion (Deering et al. 2009; Chakrabarti et al. 2003). Recent studies showed that the Polycomb group of proteins including Ezh2 that mediates H3K27me3, JMJD3 that demethylates H3K27me3, and accessory proteins such as Bmi-1 play key roles in pancreatic β-cell proliferation and regeneration through regulation of the tumor suppressor protein p16INK4a (Dhawan et al. 2009; Chen et al. 2009). Histone modifications were also reported in adipocyte differentiation since a regulatory role of H3KAc was identified in the expression of C/EBP-delta, a key transcription factor involved in adipocyte differentiation (Nakade et al. 2007). LSD1(demethylase) and SETDB1(methyltransferase) functions were required for the regulation of H3K4me2 and H3K9me2 respectively during adipogenesis (Musri et al. 2010). Furthermore, knockdown of the H3K9me2 demethylase Jhdm2a (Jmjd1a) resulted in obesity and hyperlipidemia in mice, providing direct evidence for H3Kme in the development of diabetes (Tateishi et al. 2009). The class III HDAC, SIRT1 was found to play an important role in energy metabolism, and SIRT1 activators such as resveratrol can inhibit insulin resistance. Several SIRT1 modulators are being evaluated for the treatment of insulin resistance (Haigis and Sinclair 2010; Blum et al. 2011).

19.7 Histone Modifications in Inflammatory Gene Expression

Evidence shows that NF-κB mediated inflammatory gene expression induced by pro-inflammatory signals was associated with changes in HKAc and HKme in vascular cells and monocytes. Ang II induced IL-6 expression was associated with increased NF-κB activation and promoter H3K9/14Ac in VSMC. Ang II enhanced the recruitment of TFs (NF-κB and CREB) along with co-activator HATs steroid

receptor coactivator-1 (SRC-1) and p300/ CBP to the IL-6 promoter. Furthermore, Ang II induced IL-6 expression was inhibited by a p300 mutant lacking HAT activity and a SRC-1 mutant lacking ERK phosphorylation site (Sahar et al. 2007). Oxidized lipids also increased H3KAc at the IL-6 and MCP-1 promoters in VSMC. This increased acetylation and gene expression was dependent on Src kinase activity (Reddy et al. 2009). Role of p300 and p/CAF was also reported in NF-κB mediated chemokine(C-C motif) ligand 11 (CCL11) expression in TNF-α stimulated human airway smooth muscle cells (Clarke et al. 2008).

TNF-α increased H3KAc at the IL-6 promoter, and recruitment of CBP/p300 was required for the optimal induction of NF-κB mediated IL-6 and IL-8 expression in EC (Vanden Berghe et al. 1999). TNF-α induced EC specific E-selectin expression was associated with increased H3K4me2, H3K9/14 Ac and H4K12Ac along with p300/CBP recruitment, which promoted nucleosome remodeling to increase chromatin access to NF-κB (Edelstein et al. 2005). Furthermore, these studies also showed the role of HDAC2 in association with Sin3A in the post-induction repression of these inflammatory genes. EC specific expression of endothelial nitric oxide(eNOS) was associated with H3K4me, H3K9/14Ac and Ser10-phosphorylation at the eNOS core promoter (Fish et al. 2005). Oxidized LDL could induce H3KAc, and recruitment of HATs along with NF-κB at inflammatory gene promoters in EC and these events could be reversed by statin treatment (Dje N'Guessan et al. 2009) . Together, these reports demonstrate the role of histone PTMs in regulating the expression of inflammatory genes in vascular cells.

Lipopolysaccharide (LPS) induced the expression of several HDACs along with inflammatory genes in macrophages (Aung et al. 2006). Interestingly, HDAC inhibitors attenuated the expression of some inflammatory genes but also increased the expression of pro-atherogenic genes (Halili et al. 2010). In dendritic cells derived from human monocytes, LPS induced inflammatory gene expression was associated with a rapid decrease in the levels of the repressive mark H3K9me3 followed by an increase to basal levels at later time points. This suggested a regulatory role of H3K9me3 in negative feedback mechanisms associated with post induction repression of inducible inflammatory genes (Saccani and Natoli 2002). Similar decrease in H3K9me3 at early time points and restoration to control levels at later time points was also noted in VSMC stimulated with TNF-α (Villeneuve et al. 2008). LPS mediated inflammatory gene expression was associated with decreases in repressive epigenetic mark H3K27me3 in macrophages. Further studies revealed that LPS also increased the expression of the H3K27me3 demethylase JMJD3 which was shown to regulate inflammatory genes in macrophages (De Santa et al. 2007, 2009). In THP-1 monocytes, TNF-α stimulation increased H3K4me at inflammatory gene promoters and promoted the recruitment of SET7/9, a H3K4 methyl transferase, along with NF-κB at inflammatory gene promoters. SET7/9 gene silencing revealed that a subset of NF-κB regulated inflammatory genes required SET7/9 and its mehyltransferase activity in TNF-α stimulated THP-1 cells (Li et al. 2008). TNF-α induced inflammatory gene expression was also associated with increased H3R17me and recruitment of the arginine methyltransferase CARM1 (Miao et al. 2006). Thus, NF-κB mediated inflammatory gene expression can be fine tuned by histone

modifications in both vascular cells and monocytes, key players in the pathogenesis of vascular complications.

19.8 Histone Lysine Modifications in Diabetic Vascular Complications

Several lines of evidence suggest that diabetes and diabetogenic agents promote changes in histone modifications in vascular cells and monocytes to regulate the expression of genes associated with vascular complications. HG induced increases in TNF-α and COX-2 genes in THP-1 monocytes was associated with increased H3KAc at their promoters along with increased promoter recruitment of NF-κB and co-activators CBP, p/CAF and SRC-1, and reduced occupancy of HDAC1 (Miao et al. 2004). Furthermore, increased levels of H3KAc was observed at inflammatory gene promoters in peripheral blood monocytes obtained from T1D and T2D patients, thus establishing in vivo relevance and demonstrating that inflammatory cells from diabetic patients may have a more open chromatin at pathologic genes (Miao et al. 2004). The RAGE ligand S100b also increased H3K9Ac, H3K14Ac and H3R17me as well as recruitment of p300 and CARM1 at the TNF-α promoter in THP-1 monocytes demonstrating that RAGE signaling can alter histone PTMs in chromatin (Miao et al. 2006). Recent studies reported that the anti-inflammatory agent curcumin blocked HG induced cytokine gene expression in THP-1 cells via inhibition of H3KAc and activity of p300 (Yun et al. 2011). These results further confirm the role of HKAc in inflammatory gene expression under diabetic conditions.

TNF-α induced inflammatory gene expression was enhanced in macrophages from T1D mice and this was associated with increased H3K4me and recruitment of SET7/9 at inflammatory gene promoters (Li et al. 2008). Genome-wide location approaches such as ChIP-on-chip showed that HG induced significant changes in H3K9me2 and H3K4me2 patterns at key genes in THP-1 monocytes (Miao et al. 2007). ChIP-on-chip studies using lymphocytes from T1D patients revealed significantly increased H3K9me2 levels at a subset of genes involved in inflammatory and autoimmune regulatory pathways relevant to the pathogenesis T1D and its complications (Miao et al. 2008).

Human VSMC cultured in HG displayed increases in inflammatory genes and this was associated with reduced H3K9me3 at their promoters (Villeneuve et al. 2008). In retinal EC, HG decreased global H3KAc, inhibited HAT activity and increased HDAC expression (Zhong and Kowluru 2010). Recent studies in retinal EC showed that HG mediated inhibition of the antioxidant superoxide dismutase (*sod2*) gene was associated with increased H4K20me3 at its promoter through increased expression of the corresponding HMT SUV420h2 (Zhong and Kowluru 2011). Another study reported decreases in H3K9me and increases in H3K4me along with increased recruitment of SET7/9 at fibrotic gene promoters by HG and the profibrotic growth factor TGF-β in renal mesangial cells which are involved in

diabetic nephropathy (Sun et al. 2010). Furthermore, HG induced gene expression and changes in these histone modifications were blocked by a TGF-β antibody (Sun et al. 2010). These studies show that diabetic conditions can alter the levels of histone PTMs in target cells that play key roles in diabetes and its vascular complications.

19.9 Histone Modifications in Metabolic Memory

Metabolic memory has been implicated in the persistence of vascular complications even long after achieving normal glycemic levels in diabetes patients. This was attributed to the sustained increases in the levels of oxidant stress and inflammatory genes in the vasculature (Villeneuve and Natarajan 2010; Giacco and Brownlee 2010; Pirola et al. 2010). Recent studies using cell culture and animal models have suggested that HKme may be involved in metabolic memory of diabetic vascular complications.

EC treated with HG continued to express elevated levels of inflammatory genes even long after restoration to normal glucose medium mimicking metabolic memory. There was a sustained increase in p65 (NF-κB) expression and this was associated with increased levels of the activation mark H3K4me1 and the corresponding HMT Set7/9 at the p65 promoter (El-Osta et al. 2008). In addition, levels of repressive marks H3K9me2 and H3K9me3 were also reduced while the occupancy of the demethylase LSD1 was enhanced in HG treated EC. These changes persisted even after removal of HG suggesting a key role of H3Kme and its regulators in metabolic memory in EC (Brasacchio et al. 2009). Oxidant stress and reactive dicarbonyls such as methylglyoxal were implicated in these changes (El-Osta et al. 2008).

In another model of metabolic memory, aortic VSMC isolated from *db/db* mice were used. These cells displayed enhanced expression of IL-6, MCP-1 and MCSF-1 genes and increased monocyte binding relative to *db/+* control cells, even after culturing for few passages in vitro, mimicking metabolic memory (Li et al. 2006; Villeneuve et al. 2008; Meng et al. 2010). ChIP assays showed that the repressive H3K9me3 mark was significantly reduced at the promoters of these inflammatory genes in VSMC of diabetic *db/db* mice relative to those from genetic control *db/+* mice. Furthermore, TNF-α induced inflammatory gene expression was also significantly enhanced in these *db/db* cells (Villeneuve et al. 2008) and this was associated with persistently reduced levels of repressive H3K9me3 and occupancy of the H3K9me3 methyltransferase Suv39h1 at inflammatory gene promoters suggesting dysregulation of repressive mechanisms in diabetes. Suv39h1 levels were also significantly reduced in *db/db* VSMC and reconstitution by overexpression of Suv39h1 reversed the pro-inflammatory phenotype of *db/db* VSMC (Villeneuve et al. 2008). Thus, H3K9me3 and the corresponding HMT appear to play key roles in this model of metabolic memory related to vascular complications. In addition, recent studies showed that levels of the microRNA-125b were increased in *db/db* VSMC and that Suv39h1 was a direct target of miR-125b in mouse VSMC (Villeneuve et al. 2010).

Furthermore, miR-125b could inhibit Suv39h1 expression, induce inflammatory genes, and reduce H3K9me3 at their promoters and also promote monocyte-VSMC binding in non-diabetic *db/+* cells. In contrast, miR-125b inhibition reversed key pro-inflammatory phenotypes of diabetic *db/db* VSMC (Villeneuve et al. 2010). These results identified a novel role for microRNA mediated mechanisms in inflammatory gene expression in VMSC and possibly metabolic memory through downregulation of key repressive chromatin factors. Interestingly, SIRT1 can activate the methyltransferase activity of Suv39h1 (Vaquero et al. 2007) raising the intriguing question of whether SIRT1 activators could be used for treating diabetic complications or metabolic memory.

Studies with a T1D rat model of diabetic retinopathy have suggested a role for H4K20me3 in metabolic memory (Zhong and Kowluru 2011) . There was persistently reduced expression of the key antioxidant gene *sod2* in retinas from diabetic rats with poor glycemic control that developed retinopathy compared with non-diabetic rats, or diabetic rats with good glucose control that did not develop retinopathy. This was associated with increased levels of H4K20me3, H3K9Ac as well as NF-κB recruitment at the *sod2* promoter and increases in the expression of the H4K20me3 methyl transferase Suv420h2 in diabetic retinas. Interestingly, these changes were sustained in rats displaying memory of retinopathy. Furthermore, in vitro studies with EC cultured in HG demonstrated the role of Suv420h2 in the persistent histone PTMs at the *sod* promoter. Thus, H4K20me3 and Suv420h2 were implicated in this model of metabolic memory associated with diabetic retinopathy.

19.10 Summary

Studies in cultured cells and experimental models have identified the role of HG as well as downstream biochemical, signaling and chromatin mechanisms in vascular complications and metabolic memory (Fig. 19.2). However, diabetes is a multifactorial disease and other factors such as AGEs, oxidized lipids, proinflammatory growth factors, nutrients and lifestyles could also be involved. Recent advances in genomewide approaches such as ChIP-Seq and DNA methylation profiling will greatly accelerate our understanding of histone PTMs and epigenetic mechanisms regulating the expression of genes associated with diabetic complications and metabolic memory (Wang et al. 2009; Metzker 2010; Hawkins et al. 2010; Maunakea et al. 2010). These studies could potentially lead to the identification of several other histone PTMs, HMTs, HDMs and DNA methyl transferases in diabetic complications. Since epigenetic mechanisms and histone PTMs are reversible, there is ample potential for the development of novel therapies. Several candidates including inhibitors of HDACs, HATs and DNA methyltransferases are being tested in epigenetic therapy for cancer (Kelly et al. 2010; Selvi et al. 2010). Some of these are also being considered for treating vascular complications like restenosis (Pons et al. 2009; Natarajan 2011). Recent reports showing that histone modifications and inflammatory genes can be reversed by curcumin in monocytes treated with HG

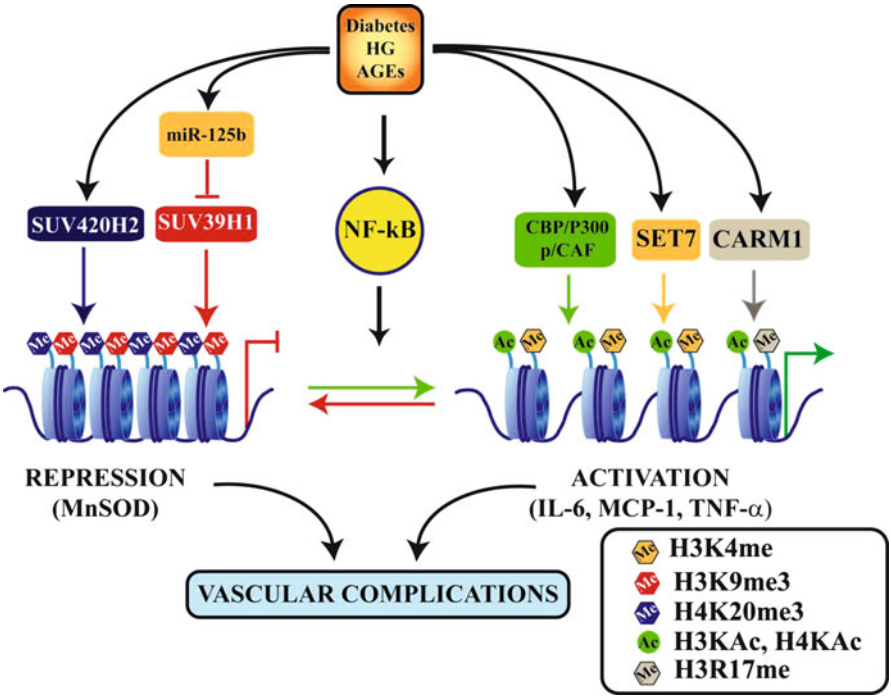

Fig. 19.2 Epigenetic mechanisms of gene regulation and metabolic memory associated with vascular complications of diabetes. Diabetes and diabetogenic agents such as high glucose (HG) and advanced glycation end products (AGEs) activate transcription factors such as NF-κB which regulate gene expression. This is further regulated by epigenetic mechanisms in chromatin activated under these conditions. These include increases in the levels of active marks such as histone H3 or H4KAc and H3K4me or H3R17me through recruitment of co-activator HATs (CBP/p300 and p/CAF) and HMTs (SET7 and CARM1) respectively. Other as yet unidentified HATs, HMTs and HDMs may also be involved. Diabetes can also lead to loss or reduction in repressive marks like H3K9me3 through reduced recruitment of the HMT SUV39H1 as well as inhibition of its expression by miR-125b, which is increased in diabetes. This leads to increased expression of inflammatory genes. In addition, diabetes can also inhibit the expression of protective anti-oxidant genes such as manganese superoxide dismutase (MnSOD) by increasing promoter levels of the repressive mark H4K20me3 and its HMT, SUV420H2. Alterations in gene expression and histone PTMs at their promoters, such as reduced H3K9me3 or increased H3K4me1 and H4K20me3, can persist even after glucose normalization demonstrating the key role of such epigenetic mechanisms in metabolic memory which leads to sustained long term diabetic complications. Ac-lysine acetylation; Me: lysine or arginine methylation

(Yun et al. 2011), and also renal dysfunction in diabetic rats (Chiu et al. 2009) by a TGF-b antibody in renal mesangial cells (Sun et al. 2010) and by statins in EC (Dje N'Guessan et al. 2009) reveal the potential of currently used drugs to directly or indirectly reverse the epigenetic mechanisms involved in diabetic vascular complications. Given the exponential increase in epigenetics research in recent years, epigenetic therapy for the treatment of diabetic complications could become a reality in the near future.

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Chapter 20
Epigenetic Changes in Inflammatory and Autoimmune Diseases

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Abstract In higher eukaryotic organisms epigenetic modifications are crucial for proper chromatin folding and thereby proper regulation of gene expression. In the last years the involvement of aberrant epigenetic modifications in inflammatory and autoimmune diseases has been recognized and attracted significant interest. However, the epigenetic mechanisms underlying the different disease phenotypes are still poorly understood. As autoimmune and inflammatory diseases are at least partly T cell mediated, we will provide in this chapter an introduction to the epigenetics of T cell differentiation followed by a summary of the current knowledge on aberrant epigenetic modifications that dysfunctional T cells display in various diseases such as type 1 diabetes, rheumatoid arthritis, systemic lupus erythematosus, multiple sclerosis, inflammatory bowel disease, and asthma.

20.1 Introduction

The number of people diagnosed with autoimmune and inflammatory diseases has increased noteworthy in the last 40 years and this fast-growing number has been impossible to explain by Mendelian inheritance only. Epigenetic modifications such

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as DNA methylation, histone modifications and chromatin remodeling are closely interwoven and constitute multiple layers of epigenetic modifications to control and modulate gene expression through their joint effects on chromatin structure (Tost 2008). The epigenome of higher eukaryotic organisms changes continuously throughout life (Calvanese et al. 2009; Whitelaw and Whitelaw 2006). In addition to the genetic influences on the epigenome, environmental factors have been found to contribute to the dynamics on the epigenome leading potentially to altered regulation of gene expression (Fraga 2009; Holliday 2006). The influence of non-genetic factors in autoimmune and inflammatory diseases has been demonstrated in studies of genetically-identical (monozygotic/MZ) twins that show a variable degree of discordance with respect to different phenotypic traits including susceptibility to these diseases. MZ twins show a concordance rate for different autoimmune diseases ranging from 12% for rheumatoid arthritis (Aho et al. 1986) up to 70% for psoriasis (Krueger and Duvic 1994). There is substantial epigenetic variation between MZ twin pairs (Ballestar 2010; Meda et al. 2011). This variation increases with age and if twins live in different environments (Fraga et al. 2005). These environment-host interactions might result in such severe epigenetic changes that the epigenetic homeostasis can no longer be maintained leading to aberrant gene expression. Aberrant gene expression in specific cells of the immune system most likely contributes to the loss of immune tolerance, inflammation and autoimmunity, which is characterized by the failure of an organism to recognize self-antigens. Multiple risk factors such as environmental toxins, drugs, hereditary factors, and viruses have been associated with the susceptibility for autoimmune diseases. However, the exact reasons for the breakdown of self-tolerance are poorly understood. Many autoimmune diseases show an increased prevalence in women. This might be possibly due to sex hormones but changes of the epigenetic landscape of T helper cells might also contribute to the higher percentage. In particular alterations in the patterns of epigenetic modifications and/or reactivation of the normally inactive X chromosome are believed to be one of the founders of the imbalance seen between the genders (Brooks 2010). Due to the growing number of incidences of these multifactorial diseases, increased attention and effort has been put in investigating the influence of aberrant epigenetic modifications.

20.2 Th Cell Differentiation

Dysfunction of T helper (Th) cells is the main precursor of autoimmunity as a shift in the balance between effector T cells and the negative regulators of the immune system (Tregs) drives the production of auto-antibodies and leads to the destruction of the respective tissues of affected individuals. T helper cells are important components of the immune system as they are required for optimal host defense against different pathogens. Their major role is to activate and direct other cells of the immune system. However, Th cells are not able to eliminate infected host cells or pathogens by themselves and would be insufficient upon infection if no other immune cells were present.

Interactions between the T cell receptor (TCR) of naïve CD4+ T cells and the major histocompatibility complex (MHC), found on antigen presenting cells (APCs), initiate a network of signaling cascades that result in differentiated effector Th cells. Depending on the mutually exclusive production of specific cytokines, differentiated Th cells can be divided into at least four major subgroups: Th1, Th2, and the more recently found lineages Th17 and regulatory T cells (Treg), reviewed in Zhu et al. (2010). The signature cytokine of Th1 cells is IFN-γ, Th2 cells are characterized by the production of interleukin 4 (IL-4), IL-5 and IL-13 whereas Th17 cells produce IL-17, IL-21, IL-22, and IL-26 (Ouyang et al. 2008). The exogenous presence of signature cytokines for Th1 and Th2 cells, IFN-γ and IL-4 respectively, is necessary for the initiation of differentiation (Le Gros et al. 1990; Lighvani et al. 2001). Th1 cells mediate immunity against intracellular pathogens whereas Th2 cells mediate humoral immunity against extracellular pathogens. Th17 cells are implicated in the defense against extracellular bacteria and fungi at epithelial and mucosal surfaces. Th cells also play a key-role in different diseases as aberrant Th1 and Th17 cytokine expression is associated with autoimmune diseases whereas allergic diseases are associated with aberrant Th2 cytokine expression (Lucey et al. 1996; Murdaca et al. 2011; Robinson et al. 1992). The negative regulators of the immune system are the Treg cells. Their main role is to maintain the homeostatic balance of immune responses through the suppression of activation, proliferation and the specific cellular function of a variety of immune cells including Th1- and Th2 cells, cytotoxic T cells, natural killer (NK) cells, B cells and antigen presenting cells (Sakaguchi et al. 2006, 2008).

For the differentiation into diverse Th cell lineages the molecular changes induced by TCR interaction are passed on to daughter cells through cell expansion. Epigenetic states play an important role when it comes to the transcriptional regulation and maintenance of a given gene. These heritable changes of the cytokine genes themselves and their regulatory elements such as enhancers, promoters, and insulators, that direct the differentiation process, have been found to start at an early stage upon TCR/MHC interaction (Ansel et al. 2003).

Several comprehensive reviews have detailed the multiple steps in Th cell differentiation (Ansel et al. 2006; Murphy and Reiner 2002; Placek et al. 2009; Wilson et al. 2009; Zhu et al. 2010), we will therefore only briefly introduce the pathways and limit this section to key-facts only. Our main focus during Th cell differentiation will be on the involvement of epigenetic modifications.

20.2.1 Th1 Cell Differentiation

IL-12 is the major Th1 differentiation initiator and is released from antigen-presenting cells (APCs) upon TCR/MHC interaction where it induces IFN-γ production through two pathways (Fig. 20.1): (1) The IL-12 induced production and secretion of IFN-γ from natural killer cells activates the transcription factor STAT1 once IFN-γ has bound to its receptor IFNGR. STAT1 activation leads in turn to the expression of the

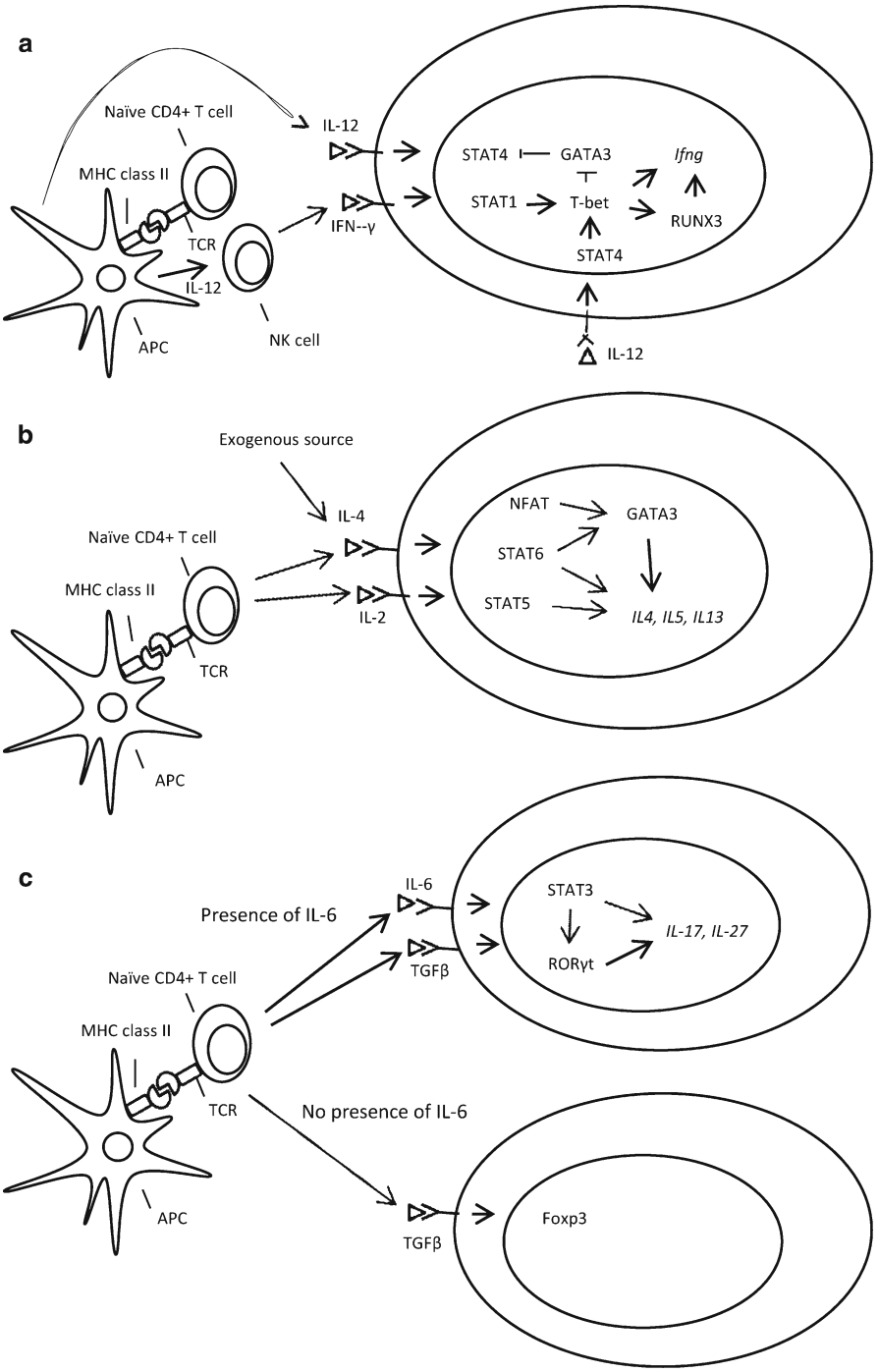

Fig. 20.1 Key cytokines and transcription factors involved in Th cell differentiation. (a) Differentiation of the Th1 cell linage is initiated by TCR/MHC engagement. As IL-12, the major differentiation initiator is released from APCs the transcription of *INF-γ* is achieved through two pathways. The first includes the STAT1 induced transcription of T-bet, the key-regulator of Th1 differentiation.

transcription factor T-bet (Afkarian et al. 2002; Weaver et al. 2007) a key-regulator of the Th1 lineage that regulates multiple genes including *Ifng* (Djuretic et al. 2007), induces the up-regulation of *Runx3* (Djuretic et al. 2007), and negatively regulates GATA3 (Usui et al. 2006). A collaboration between T-bet and Runx3 is further observed as they together have a positive regulatory effect on *Ifng* and a negative regulatory effect on *Il-4* (Djuretic et al. 2007; Naoe et al. 2007). GATA3 is a T cell specific transcription factor with counteracting function on Th1 differentiation as it down-regulates STAT4 (Usui et al. 2003, 2006) the other key-regulator of Th1 differentiation (Fig. 20.1). (2) In the second IL-12 induced Th1 differentiation pathway, IL-12 binds to its cell-surface receptor, IL-12R, which is composed of the two subunits IL-12Rβ1 and IL-12Rβ2 (Presky et al. 1996). The expression of IL-12Rβ2 is dependent on the Brahma-related gene 1 (BRG1) product, which is recruited to the promoter and enhancer of *IL-12Rβ2* rapidly after TCR engagement in resting peripheral T cells (Letimier et al. 2007). BRG1 is the ATPase subunit of the SWI/SNF-like BAF (Brahma-related gene (BRG1)/Brahma (BRM)-associated factor) chromatin remodeling complex (BAF complex) and the recruitment of the BAF complex is associated with histone hyperacetylation and low-level transcription of *IL-12Rβ2* (Letimier et al. 2007). Translocation of the newly synthesized IL-12Rβ2 subunit to the cell surface completes the composition of the IL-12 receptor through which IL-12 induces the activation of STAT4 (Thieu et al. 2008). STAT4 expression has a positive feedback mechanism resulting in high-levels of *IL-12Rβ2* transcription (Letimier et al. 2007) and inducing in turn STAT4 and thereby IFN-γ production. Furthermore, there is *in vitro* evidence that STAT4 positively regulates T-bet at the transcriptional level (Yang et al. 2007) (Fig. 20.1).

20.2.2 Th2 Cell Differentiation

Upon TCR engagement, IL-4 induces the activation of STAT6, which activates GATA3 in cooperation with several TCR-induced signals including the nuclear factor of activated T cells (NFAT) (Ouyang et al. 2000; Zhu et al. 2001) (Fig. 20.1). STAT6 and GATA3 induce synergistically the production of the signature cytokines

Fig. 20.1 (continued) In co-operation with Runx3, T-bet up-regulates INF-γ production. The other pathway includes the STAT4 induced transcription of *INF-γ*. GATA3 is a negative regulator of the Th1 cell lineage as it suppresses STAT4. (**b**) Subsequent to TCR/MHC engagement, IL-4 is the major differentiation initiator of Th2 differentiation. IL-4 activates STAT6 and in co-operation with NFAT they induce the activation of GATA3. Together STAT6 and GATA3 result in the production of the Th2 signature cytokines; IL-4, IL-5, and IL-13. The STAT5 induced production of Th2 specific cytokines is initiated as IL-2 binds to its receptor and is GATA3 dependent. (**c**) Differentiation into Th17- and Treg cells is initiated by TGF-β, whereby the presence of IL-6 is decisive in which lineage the cells will differentiate. If present in the external milieu, RORγt will be expressed and the differentiation will go towards Th17 cell linage. In the absence of IL-6 the key transcription factor of Treg differentiation, FOXP3, inhibits the expression of RORγt and encourages differentiation towards Treg cell lineage

IL-4, IL-5, and IL-13. Failure of Th2 differentiation in mice upon *Gata3* knockout in peripheral T cells strongly indicates the importance of Gata3 as a key-regulator of Th2 development (Pai et al. 2004; Zhu et al. 2004). Moreover, the presence of Gata3 is also important for the Stat5 induced production of Il-4 as they collaborate in priming naïve Th cells for the Th2 phenotype (Zhu et al. 2003). Stat5 becomes activated after IL-2 binds to its receptor. GATA3 is thus involved in the Th2 differentiation in both an IL-4 dependent and in an IL-2 dependent manner (Fig. 20.1). GATA3 has an auto-activation effect and stabilizes together with the autocrine and paracrine IL-4 induced activation of STAT6 the Th2 lineage development (Ansel et al. 2006).

20.2.3 Th17 and Treg Cell Differentiation

The differentiation process for these two Th cell lines is initiated by the transforming growth factor β (TGFβ), which simultaneously blocks Th1- and Th2 cell differentiation. The fate of cell differentiation depends on the presence of IL-6 (Kimura and Kishimoto 2010). Il-6 induces in cooperation with TGF-β the expression of the steroid receptor-type nuclear receptor (RORγt), which is required and specific for Th17 development (Ivanov et al. 2007; McGeachy and Cua 2008) (Fig. 20.1). RORγt and STAT3 drive the production of the Th17 signature cytokines; IL-17, IL-21, IL-22, and IL-26. In addition, IL-6 inhibits the expression of FOXP3 through IL-6 induced STAT3 activation (Yang et al. 2008). In the absence of IL-6 the key-transcription factor of Treg differentiation, FOXP3, inhibits RORγt and thereby Th17 differentiation and encourages instead Treg cell line engagement (Ivanov et al. 2007).

20.2.4 Epigenetics and Th Cell Differentiation

Major changes to the patterns of epigenetic modifications within the *IFN-γ* and *IL-4* loci are driven by TCR engagement. Once established these heritable changes in gene expression are passed on to daughter cells where they contribute to Th cell maintenance. Specific histone modifications are associated with heterochromatin and euchromatin (Kouzarides 2007). Heterochromatin, in which the DNA is densely wrapped around the octamer of histone proteins, is associated with modifications such as H3K9-, H3K27-, and H4K20 methylation. On the contrary, euchromatin is associated with epigenetic modifications such as H3K4 methylation and acetylation of lysine residues of H3 and H4.

To permit a prompt and specific response upon infection with a pathogen, naïve T cells have to be poised to enable rapid differentiation in either direction in function of exogenous stimuli. Interestingly, poised $CD4^+$ T cells have been shown to express low levels of both IFN-γ and IL-4 (Grogan et al. 2001) and the histones located at the genes encoding the cytokines display a bivalent histone modification pattern. They thus display a combination of histone modifications normally associated

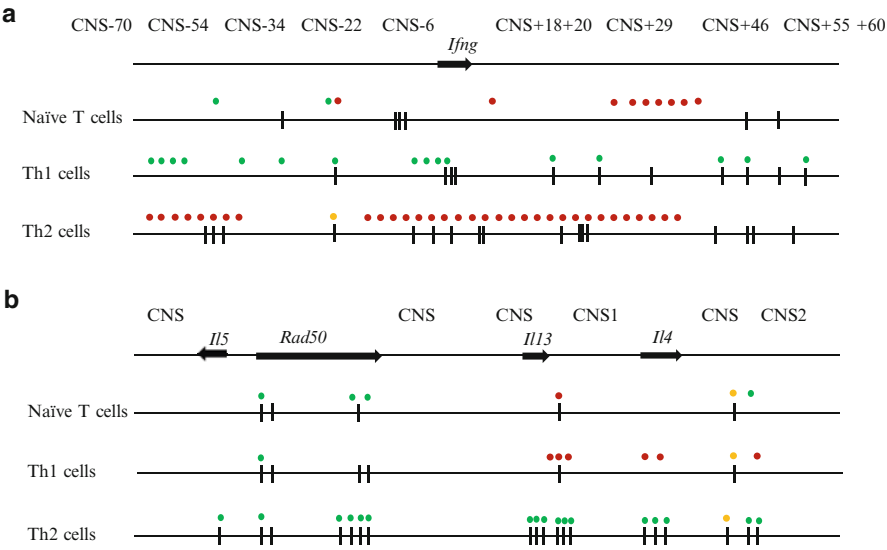

Fig. 20.2 The epigenetic state of the Ifng- and Il-4 locus during Th cell differentiation. Depending on the external cytokine milieu upon TCR/MHC engagement different epigenetic modifications can be found throughout the *Ifng*- and *Il-4* locus. The black bars indicate hypersensitive sites, green dots indicate permissive epigenetic modification, red dots indicate repressive epigenetic modifications, and yellow dots indicate bivalent epigenetic modifications. (**a**) During Th1 cell differentiation the *Ifng* locus is associated with permissive epigenetic modifications contradictory to the *Ifng* locus during Th2 cell differentiation. CNS-22 is believed to be an important regulatory region as consensus binding sites for the key-transcription factors, T-bet, GATA3, STAT4, STAT6, NF-κB, and Ikaros, are found here together with histone hyperacetylation in both naïve-, Th1-, and Th2 cell lineages. (**b**) The *Il-4* locus is associated with permissive epigenetic modifications during Th2 cell differentiation. During Th1 cell differentiation the *Il-4* locus is mostly associated with repressive epigenetic marks

with active transcription (H3K4me2 and H3K9/14 acetylation) combined with modifications normally associated with repressive transcription (H3K27me3) within the two loci (Avni et al. 2002; Baguet and Bix 2004; Fields et al. 2002). High DNA methylation levels (~90%) within the *IL-4* locus contribute to the restrained Th2 cytokine expression in naïve T cells (Lee et al. 2002; Makar et al. 2003). However, the *IL-4* promoter is less methylated in undifferentiated T cells facilitating the observed low-level transcription of the gene (Makar et al. 2003).

Cis-regulatory elements for the genes encoding the cytokines have been identified by *in silico* approaches as conserved non-coding regions (CNSs) (Nardone et al. 2004) or experimentally as DNase I hypersensitivity sites (HSs) (Schoenborn et al. 2007). A region encompassing ~130 kb constitutes the regulatory platform for *IFN-γ* (Barski et al. 2007; Boyle et al. 2008) and within this region several CNSs have been identified (Balasubramani et al. 2010; Hatton et al. 2006; Schoenborn et al. 2007; Shnyreva et al. 2004) (Fig. 20.2). The regulatory region for the *IL-4* locus encompasses a ~85 kb region containing *IL-4*, *IL-5*, *IL-13*, and the ubiquitously expressed

Rad50 which is involved in DNA repair. Six CNSs have been identified within the *IL-4* locus together with a number of HSs (Loots et al. 2000; Nardone et al. 2004; Takemoto et al. 1998; Wilson et al. 2009; Yamashita et al. 2002) (Fig. 20.2).

20.2.5 Epigenetic Changes Within the IFN-γ Locus During Th Cell Differentiation

Several epigenetic marks associated with actively transcribed genes are found throughout the *IFN-γ* locus rapidly after IL-12 and IFN-γ induced Th1 differentiation. One of these permissive epigenetic marks is acetylation of lysines of H3 at individual CNSs (Zhou et al. 2004). The key-transcription factor controlling Th1 differentiation, T-bet, is also involved in the epigenetic regulation through additional events including H4 acetylation at regulatory sites located +20, +30, +46, +50, and +60 kb upstream of the transcription start site of *Ifng*. T-bet further recruits the jumonji-domain-containing protein histone demethylase 3 (JMJD3) which is a part of the H3K27 demethylase complex (Miller et al. 2008). Simultaneously, T-bet associates with the retinoblastoma-binding protein 5 (RbBp5) resulting in H3K4 methyltransferase activity facilitating access to the DNA (Miller et al. 2008). These observations suggest the importance of T-bet in reversing repressive epigenetic marks within the *INF-γ* locus. Permissive marks are also found at the level of histone methylation. H3K4 dimethylation, normally associated with active transcription, is found within the *IFN-γ* locus from CNS +20 to CNS +60 contributing to active transcription of *IFN-γ* (Hamalainen-Laanaya et al. 2007). However, not only permissive marks are found throughout the *IFN-γ* locus during Th1 differentiation. H3K9 dimethylation, normally associated with silenced genes, is also present during the differentiation process (Chang and Aune 2007). These conflicting epigenetic marks of the actively transcribed *IFN-γ* locus may serve as a regulatory mechanism to ensure proper IFN-γ production.

The two key-transcription factors of Th2 differentiation, STAT6 and GATA3, are involved in the establishment of repressive epigenetic marks, notably H3K27 di- and trimethylation, throughout the *IFN-γ* locus (Fig. 20.2). The binding of STAT6 to the *Ifng* promoter is involved in the establishment of the characteristic repressive mark, H3K27 trimethylation (Chang and Aune 2007). GATA3 also binds the *Ifng* promoter to which it recruits EZH2, a component of the Polycomb repressive complex 2, that is associated with H3K27 trimethylation and the formation of heterochromatin (Chang and Aune 2007). Thus during Th2 differentiation GATA3 is associated with both transcriptional activation of genes encoded within the *IL-4* locus and transcriptional repression of *Ifng*. It has been proposed that it is the number of GATA-3 binding sites within the two loci, *IFN-γ* and *IL-4* respectively, which will determine the differences of epigenetic marks and thereby the different outcome for GATA3 binding (Aune et al. 2009).

The multi-functional transcriptional regulator CCCTC-binding factor (CTCF) is implicated in Th2 differentiation as it has been found that transcription of IL-4, IL-5, and IL-13 was strongly reduced in CTCF-deficient Th2 cultures (Ribeiro de Almeida et al. 2009). Interestingly, CTCF-deficiency only had moderate influence on

IFN-γ production and the IL-17 production in Th17 cells was unaffected (Ribeiro de Almeida et al. 2009).

Individual CNSs have important influences on Th cell differentiation. Early remodeling of the *Ifng* locus is suggested to be dependent on CNS −6 (Fig. 20.2) as this region possesses a DNase I hypersensitive site in naïve T cells (Bream et al. 2004; Lee et al. 2004). CNS −22 is believed to be an important regulatory region as it displays a high concentration of consensus binding sites for key-transcription factors involved in T cell development and differentiation such as T-bet, GATA3, STAT4, STAT6, nuclear factor-κB (NF-κB), and Ikaros. Furthermore, histone hyperacetylation in this region has been found in both naïve T cells as well as in Th1 and Th2 cells indicating a key-regulatory effect for *IFN-γ* transcription (Hatton et al. 2006). Insulators of the *Ifng* locus have been mapped to CNS −54 and CNS +46 (Schoenborn et al. 2007).

20.2.6 Epigenetic Changes Within the IL-4 Locus During Th Cell Differentiation

During Th1 differentiation the permissive epigenetic marks of the *IL-4* locus, found in naïve T cells, have to be erased for definite IFN-γ expression. The methyl CpG-binding domain 2 (MBD2) may serve an important function in IL-4 silencing as it has been shown that deficiency of this protein results in heritable aberrant IL-4 expression in Th1 cells lacking GATA3 (Hutchins et al. 2002). MBD2 is known to associate with histone deacetylase (HDACs) complexes resulting in the formation of heterochromatin. H3K27 tri-methylation contributes to silencing of the *IL-4* locus as well (Koyanagi et al. 2005; Wei et al. 2009).

The active *IL-4* locus displays H3K4 tri-methylation (Wei et al. 2009) and the responsible histone methyltransferase MLL has been found to be recruited to *Gata3* and *Il-4* where it serves to maintain the permissive mark throughout cell division (Yamashita et al. 2006). Interestingly, the function of MLL seems to be specific for Th2 cells as Th1 differentiation was not affected in haplo-insufficient mice (Yamashita et al. 2006). GATA3 itself has also been found to be involved in the regulation of several epigenetic modifications as it assists in the formation of accessible DNA by association with H3K4 methyltransferases and recruitment of histone acetyltransferases (HATs) (Ansel et al. 2006; Yamashita et al. 2006). Furthermore GATA3 can displace bound MBD2 and the associated HDAC complexes responsible for the establishment and maintenance of heterochromatin (Hutchins et al. 2002). Additionally GATA3 can displace Dnmt1 thus inhibiting DNA methylation (Makar et al. 2003; Tykocinski et al. 2005). Epigenetic changes are not only seen on the level of histone modifications. DNA methylation is also implicated in Th2 differentiation as the high DNA methylation level in the 5′ region of the *IL-4* gene, in naïve T cells, as well as the hypermethylated intergenic region between *IL-4* and *IL-13* become specifically demethylated in a replication-dependent manner (Lee et al. 2002). The DNA demethylation is strongly associated with high-levels of IL-4 transcription (Lee et al. 2002).

20.3 Epigenetics in Autoimmune and Inflammatory Diseases

Autoimmune- and inflammatory diseases are characterized by a failure of the capability of the immune system to recognize and tolerate the body's own molecular components. In the following paragraphs we will summarize the current knowledge on epigenetic changes in these diseases. Virus associated inflammatory diseases that display profound epigenetic changes such as gastritis or cirrhosis will not be mentioned within this chapter.

20.3.1 Type 1 Diabetes

Type 1 diabetes (T1D) is a complex autoimmune disease affecting more than 30 million people worldwide. It is caused by a combination of genetic and non-genetic factors leading to immune destruction of insulin-secreting islet cells (Eizirik et al. 2009).

A microarray study by Miao *et al*. established the presence of an aberrant histone methylation profile in lymphocytes of T1D patients (Miao et al. 2008). They identified a cluster of genes with increased H3K9me2, normally associated with transcriptional repression. Many of the genes with increased H3K9me2 were involved in cytokine signaling pathways or encoded transcription factors (Miao et al. 2008). In particular increased H3K9me2 within the promoter region of *CTLA4* revealed a direct link between T1D and aberrant epigenetic modifications in lymphocytes as CTLA4 is involved in repressive T cell response.

In addition dysfunction of Treg cells has been suggested to play a role in T1D pathogenesis (Sgouroudis and Piccirillo 2009). *In vivo* studies support this suggestion as the expression of Foxp3, the key-transcription factor of Treg cell differentiation was induced upon treatment with the DNMT inhibitor decitabine in NOD (non-obese diabetic) mice and the induction was associated with a significant demethylation of the *Foxp3* CpG island (Zheng et al. 2009). DNA methylation also plays a role in the upregulation of LFA-1 as a consequence of hypomethylation of $CD4^+$ T cells which is characteristic for auto-reactive T cells (Richardson 1986). LFA-1 co-stimulates the TCR/MHC interaction (Wulfing et al. 2002), but when overexpressed LFA-1 is thought to stabilize even TCR/MHC interaction with low-affinity and thereby contribute to autoimmune response.

20.3.2 Rheumatoid Arthritis

Rheumatoid arthritis (RA) is an autoimmune disease affecting ~ 1% of the population caused by a combination of genetic predisposition, deregulated immunomodulation and environmental influences and characterized by chronic inflammation and

destruction of synovial joints (Firestein 2003). Global DNA hypomethylation of peripheral blood mononuclear cells (PBMCs), synovial mononuclear cells and synovial tissue isolated from patients diagnosed with RA is likely to cause the aberrant expression of pro-inflammatory genes (Corvetta et al. 1991; Karouzakis et al. 2009; Liu et al. 2011; Neidhart et al. 2000). Activation of the normally inactivated retrotransposable element LINE-1 has been observed in the synovial fluid pellet from RA patients. This is thought to be a consequence of the global hypomethylation that might result in genomic instability. Activated LINE-1 elements can integrate themselves in the genome and affect gene expression and are therefore thought to contribute to the RA phenotype (Neidhart et al. 2000).

Repressed expression of the death receptor 3 (DR3) in synovial cells has been associated with hypermethylation of its promoter region (Takami et al. 2006). This finding provides a direct link between an epigenetically regulated gene and the RA phenotype as *DR3* is a member of the apoptosis-inducing Fas family. DR3 can initiate the intrinsic apoptotic network (Ashkenazi and Dixit 1998). Thus down-regulated DR3 expression contributes to the resistance of synovial cells to undergo apoptosis, which is a hallmark of RA.

Histone deacetylase inhibitors (HDACi) have gained increased interest for the treatment of RA, as they have been associated with decreased levels of pro-inflammatory cytokines (see Table 20.1). At the same time a loss of HDAC1 and HDAC2 activity in synovial tissue from RA patients has been observed compared to normal synovium (Huber et al. 2007). This seems contradictory to the promising strategies using HDACi's for RA treatment in mouse and rat models. However, it is unknown in which specific cell types the HDAC activity is decreased. It might be possible that HDACs are upregulated in other cell types and thereby make these cells possible targets for treatment. In addition, the large number of proteins, which can be modulated by HDACs and the possible overlap of targets between HDACs of the same class, makes it difficult to predict and discern the specific molecular effects of HDAC inhibitors.

20.3.3 Systemic Lupus Erythematosus

Systemic lupus erythematosus (SLE) is a chronic autoimmune disease which is characterized by the overproduction of autoantibodies against nuclear self-antigens leading to persistent damage in multiple tissues (Sawalha and Harley 2004).

Lupus is probably the best-studied autoimmune disease at the level of epigenetic modifications and especially DNA methylation. Decreased expression and activity of DNMTs in SLE patients (Richardson et al. 1990) causes a global DNA hypomethylation, which contributes to the aberrant gene expression of a large number of pro-inflammatory genes (Javierre et al. 2010). Very recently it was shown that two miRNAs (miR-21 and miR-148) promote the observed hypomethylation

Table 20.1 The outcome of using HDACi's in different models resembling inflammatory and autoimmune diseases

Disease	Model	Name of HDACi	Effect	Reference
Autoantibody-mediated arthritis	Mouse	FK-228	Decreased expression of TNF-α and IL-1β	Nishida et al. (2004)
Collagen-induced arthritis	Mouse and rat	SAHA and MS-275	Reduced arthritis scores	Lin et al. (2007)
Autoantibody-mediated arthritis	Mouse	Trichostatin A	Reduced arthritis scores and expression of matrix-degrading enzymes	Huber et al. (2007)
Systemic lupus erythematosus	*In vitro* cultured SLE T cells	Trichostatin A	Down-regulating CD154 and IL-10 while up-regulating IFN-γ	Mishra et al. (2001)
Systemic lupus erythematosus	Mouse	Trichostatin A	Up-regulates Treg cells	Reilly et al. (2008)
Inflammatory bowel disease	Mouse	Valproic acid and vorinostat (SAHA)	Suppression of pro-inflammatory cytokines and increased H3 acetylation Increased apoptosis of lamina propria lymphocytes	Glauben et al. (2006)
Inflammatory bowel disease	Mouse	Trichostatin A	Increased acetylation of Foxp3 and thereby the Treg mediated suppression of inflammation	Tao et al. (2007)

by targeting the DNA methyltransferase 1, interfering thus with maintenance of DNA methylation (Pan et al. 2010). It is also noteworthy, that lupus-like symptoms can also be induced by pharmaceutical regimens such as the vasodilator hydralazine used for the treatment of hypertensive disorders and procainamide administered for the treatment of cardiac arrhythmias. These two non-nucleoside compounds do have demethylating capabilities, although they are much less effective compared to the nucleosides analogues. There are strong analogies in the expression patterns of T-cells treated with demethylating agents such as 5-azacytidine and a subset of T-cells found in patients with active lupus. These methylation-sensitive genes that become hypomethylated upon chemical treatment or during the disease process include notably *CD11a* (*ITGAL*), *CD70*, *PRF1* and *CD40LG*. *CD11a* and *CD70* are both found overexpressed in Th cells (Zhao et al. 2010a, b), which might be due to the global hypomethylation. CD11a is the alpha chain of the integrin LFA-1 which is associated with cell adhesion and co-stimulatory signaling (Wulfing et al. 2002). CD70 is the ligand of the cell-surface receptor CD27, which plays a role in long-term maintenance of T cell immunity and the regulation of B cell activation. Decreased activity of the transcription factor regulatory factor X 1 (RFX1) in Th cells influences the epigenetic state of the promoter region of CD11a and CD70 as RFX1 is involved in the recruitment of DNMT1 and HDAC to the respective promoter region. Decreasing levels of RFX1 are thus associated with hypomethylation and hyperacetylation of the promoters of CD11a and CD70 thus increasing the transcription levels of these autoimmune genes (Zhao et al. 2010a). RFX1 further recruits the histone methyltransferase SUV39H1 as decreased levels of the transcription factor were associated with reduced H3K9 trimethylation within the same region (Zhao et al. 2010b). A strong gender bias exists among SLE patients with a female to male ratio of 9:1 (Soto et al. 2004). CD40LG is encoded on the X-chromosome, one of which is normally silenced in females through multiple (epigenetic) mechanisms. DNA demethylation in Th cells of SLE patients results in expression of the normally inactivated *CD40LG* in women (Lu et al. 2007) and might thereby contribute to the excess production of autoantibodies as CD40LG is a B cell co-stimulatory molecule (Zhou et al. 2009). DNA hypomethylation of the normally inactivated X-chromosome in women is therefore thought to contribute to the female predominance associated with SLE.

Analysis of DNA methylation patterns in MZ twins discordant for the disease phenotype revealed lower methylation levels of rRNA encoding regions in SLE patients (Javierre et al. 2010). As a consequence of the lower DNA methylation levels an overexpression of rRNA was observed which might result in increased assembly of ribosomal complexes that could trigger the autoantibody-production (Javierre et al. 2010).

Although DNA methylation is the best studied epigenetic mechanism in SLE patients an *in vitro* study found that aberrant expression of CD154, IL-10, and IFN-γ could be reversed by treating cells with the HDACi trichostatin A (Mishra et al. 2001). Further, the use of TSA proved to modulate SLE-like disease in mouse model by up-regulating Treg cells (Reilly et al. 2008).

20.3.4 Multiple Sclerosis

Demyelination of the central nervous system (CNS) caused by chronic inflammation is the hallmark of multiple sclerosis (MS).

Hypomethylation of *PAD2*, a gene encoding the peptidyl arginine deiminase type II protein that is responsible for the conversion of the guanidine group of arginines residues into citrulline, results in overexpression of the corresponding gene in MS patients (Mastronardi et al. 2007). The substrate of PAD2 is the myelin basic protein (MBP) but when abnormally citrullinated a conformational change of MBP occurs and it can no longer generate normal myelin (Moscarello et al. 1994).

20.3.5 Inflammatory Bowel Disease

Crohn's disease (CD) and ulcerative colitis (UC) both belong to a heterogeneous group of diseases named idiopathic inflammatory bowel disease (IBD). Despite the early stage of epigenetic studies in IBD, studies have indicated a potentially central role of histone acetylation as improvement of inflammatory conditions has been observed upon HDACi treatment. Inhibition of HDACs suppressed the production of pro-inflammatory cytokines, induced apoptosis, and caused increased acetylation (Glauben et al. 2006). Inhibition of HDACs further resulted in enhanced Treg mediated suppression of IBD *in vivo* indicating the broad implications of histone acetylation in IBD pathogenesis (Tao et al. 2007). The upregulated Treg mediated suppression of inflammation was associated with increased acetylation in the forkhead domain of Foxp3 resulting in its upregulation (Tao et al. 2007). Together these studies indicate that the formation of heterochromatin and thereby induced gene silencing is crucial for IBD pathogenesis. Interestingly, Kryczek *et al*. found that Treg cells can induce the production of IL-1 and IL-6 in colitic cells. The latter finding combined with the presence of IL-17 expressing T cells indicates that a subgroup of T cells combining the Treg and Th17 phenotype might actively contribute to the progression towards early tumorigenesis in ulcerative colitis (Kryczek et al. 2011).

Interestingly it has recently been suggested that treatment of IBD with HAT inhibitors might provide an opportunity to improve inflammatory conditions as well as H4 acetylation was significantly upregulated in the inflammatory tissue of patients diagnosed with CD (Tsaprouni et al. 2011).

20.3.6 Asthma

Asthma is characterized by an inflammatory obstruction of the respiratory airways. DNA methylation is the most extensively studied epigenetic mechanism and has been proposed as a biomarker for environmentally related asthma. Exposure

to diesel exhaust particles (DEP) has been shown to change the overall DNA methylation profile in healthy elderly people (Baccarelli et al. 2009) and DNA methylation of the *ACSL3* 5′- CpG island may indicate the extent of transplacental exposure to DEPs (Perera et al. 2009). More specifically, DEP exposure induces hypermethylation of specific sites within the *INF-γ* promoter simultaneously with hypomethylation within the *IL-4* promoter in mice. These epigenetic changes correlate with IgE production (Liu et al. 2008). This most likely contributes to the excessive amounts of Th2 signature cytokines; IL-4, IL-5, Il-9, and Il-13 associated with this chronic disease of the respiratory airways (McGee and Agrawal 2006; Zhu and Paul 2008).

Recently, hypermethylation of *Foxp3*, the key transcription factor required for Treg commitment, was found to be a consequence of ambient air pollution (AAP) exposure in children (Nadeau et al. 2010). The hypermethylation of *Foxp3* was associated with impaired Treg-mediated repression of the overactive immune response. This finding further supports the increasing evidence for a major role of Treg cells in the pathogenesis of asthma (Durrant and Metzger 2010; Larche 2007; Taylor et al. 2005).

The knowledge on the precise influence of aberrant histone modification in asthma is still very limited. However, transcription of the pro-inflammatory genes may be affected by aberrant acetylation of the N-terminal histone tails as increased HAT and reduced HDAC activity have been observed in patients diagnosed with asthma (Cosio et al. 2004; Hew et al. 2006). Natural microbial exposure represents an important environmental component for asthma protection. In a murine model, pregnant mother mice were exposed to the non-pathogenic Gram-negative bacterium *Acinetobacter Iwoffii F78* (Brand et al. 2011). Prenatal *A. Iwoffii* F78 administration prevented the development of the asthma phenotype in the progeny in an IFN-γ dependent manner. The *IFN-γ* promoter revealed a significant increase in histone H4 acetylation whereas the *IL-4* promoter showed a significant decrease, which closely correlated with IFN-γ and IL-4 RNA/protein levels in $CD4^+$ T-cells in the offspring. Treatment with the histone acetyltransferase inhibitor Garcinol abolished the asthma protective phenotype paralleled by inhibition of H4 acetylation of the *IFN-γ* promoter. These data provide evidence for the importance of epigenetic regulation of TH1/TH2 cytokine genes in transmaternal asthma protection and provides provide a molecular explanation for the protective effects of microbial exposure for the development of the asthma phenotype (Brand et al. 2011).

However, the establishment of direct links between epigenetic states and the disease phenotype is difficult and hampers the use of epigenetic modifications as reliable biomakers: (1) It is so far unknown in which specific cell type epigenetic alterations can be best correlated with the asthma phenotype; are these cells of the immune or of the respiratory system. (2) In the latter case the access to the proper type of tissue may be difficult as it might require unnecessary surgical procedures. (3) Asthma is a heterogeneous group of diseases comprising undoubtedly several clinical phenotypes with similar symptoms but different disease etiology. It is therefore probable that the epigenetic landscape would be different from phenotype to phenotype.

20.4 Epigenetic Therapy of Autoimmune Diseases

The involvement of aberrant regulation of Th cells in autoimmune and inflammatory diseases is today well accepted. Especially the emergence of the two more recently found Th lineages Th17 and Treg has increased our understanding of the process leading towards autoimmunity as a disruption of the homeostatic balance between these two cell types is crucial for the autoimmune conditions (Buckner 2010; Selmi 2009). Very recent findings regarding the plasticity of Treg and Th17 cells might potentially add a new layer of complexity to the common idea that Tregs act solely as autoimmune- and inflammatory repressors as a newly discovered subset of T cells, possessing a mixed phenotype of Treg and Th17 cells, has been suggested to contribute to autoimmune disease instead of counteracting the development (Kryczek et al. 2011). This subset of T cells is believed to develop in inflammatory microenvironments and might constitute a special effector T cell population as they express IL-2, INF-γ, and TNF-α. The phenotypic shift from Treg cells towards Th17 cells has also been shown to take place in skin lesions of patients diagnosed with severe psoriasis (Bovenschen et al. 2011). Interestingly, by treating Treg cells, isolated from severe psoriasis patients, with the HDAC inhibitor TSA they prevented the Th17-like phenotype. This observation has earlier been documented in Tregs isolated from human blood (Koenen et al. 2008) indicating the possibilities for epi-drugs to prevent the expression of pro-inflammatory cytokines generated due to this shift.

Understanding the impact of epigenetics in these multifactorial diseases leads to possibilities for the use of epigenetic drug treatments of affected individuals such as DNA demethylating agents or regimens interfering with some forms of histone modifications. Despite the early stage of epigenetic investigations within the field of autoimmune and inflammatory diseases, interest has focused on the opportunities for reversing patterns of histone acetylation as a number of *in vitro* and *in vivo* studies reported improvements of autoimmune diseases upon HDACi treatment (see Table 20.1). Some of the most widely used HDAC inhibitors for anti-autoimmunity and inflammatory responses are SAHA (Vorinostat), TSA (Trichostatin A), and valproic acid (Table 20.1). SAHA and TSA belong to the group of hydroxamic acids that inhibit zinc-dependent HDACs. With their zinc-binding group (ZBG) hydroxamic acids chelate the zinc ion near the bottom of the cylindrical pocket of HDACs. Opposite the zinc ion they have a capping group that interferes with the amino acid residues lining the entrance of the cylindrical pocket. Thereby the inhibitors block the entrance for other HDAC targets thus inhibiting its function. Along effects on non-histone proteins, this results in histone acetylation and gene expression. Valproic acid is a short-chain fatty acid thus belonging to another group of HDAC inhibitors than the two mentioned above. However like SAHA and TSA, valproic acid also interferes with HDACs catalytical domain and renders substrate recognition impossible, reviewed in (Kristensen et al. 2009). Dysfunction of HATs is thought to be the reason for the increased levels of histone acetylation observed in autoimmune and inflammatory diseases. Especially the two HATs, CBP and PCAF, have been found to be translocated to genes encoding inflammatory factors

and thereby influencing their expression. This led to the suggestion that HAT inhibitors could be applied as a new treatment strategy. HATs are diverse and can be divided into three groups: (1) The GNATs (Gcn5 N-acetyltransferases), which include p300/CBP-associated factor (PCAF), Elp3, Hat1, Hpa2, and Nut1. (2) The MYSTs containing a 60 kDa Tat interactive protein including Morf, Ybp2, Sas2, and Tip60. (3) The third group includes the orphan HATs, which do not contain a precise consensus HAT domain. Naturally occurring as well as synthesized HAT inhibitors have been investigated for their capability to decrease the activity of HATs and to what extent they can be used without severe toxicity (Arif et al. 2009; Lau et al. 2000; Stimson et al. 2005). P300 is the most investigated HAT and can be inhibited in a competitive and non-competitive manner (Arif et al. 2009). A hydroxygroup was found to be important for the binding of two competitive inhibitors (garcinol and garcinol based derivatives) to the catalytical acetyl-CoA binding site. However, binding of the acetyl-CoA binding site was not a requirement for the inhibition of p300 as monosubstitution of the C-14 position of isogarcinol created a highly noncompetitive inhibitor of p300 binding to a site outside the acetyl-CoA site but nonetheless decreasing HAT activity (Arif et al. 2009). The importance of HAT inhibitors has been reviewed in (Selvi et al. 2010).

HDAC inhibitors have been shown to suppress the production of pro-inflammatory cytokines (Glauben et al. 2006) even at a significantly lower doses compared to the concentrations used in other diseases such as cancer. This finding might be of great importance as appropriate diet might have a preventive quality for chronic inflammatory disorders (vel Szic et al. 2010). For example, high levels of sulphoraphane, an HDACi naturally found in broccoli sprouts has been associated with H3 and H4 hyperacetylation. Moreover, sulphoraphane has been associated with both global and localized hyperacetylation, G_2/M cell cycle arrest and increased apoptosis in colon cancer (Myzak and Dashwood 2006). However, precaution has to be taken into account as some HDACis have also been found to induce the expression of pro-inflammatory cytokines. The unpredictable outcome of HDAC inhibitors may be associated with the large number of targets for HDACs. Histone proteins as well as non-histone proteins regulated by HDACs are involved in different cellular pathways. Thus, depending on the HDACi of choice, a different outcome might be expected. Therefore it is crucial to further investigate the different HDAC isoforms and their specific targets to ensure the best treatment strategies.

The reactivation of Treg cells in autoimmune diseases has also been thought to be a reasonable therapeutic approach (Buckner 2010) as it has been shown that HDAC inhibitors can promote the generation and function of Treg cells (Tao et al. 2007). Especially inhibition of HDAC9 proved to be important in the regulation of Foxp3-dependent suppression of Il-2 (Tao et al. 2007). This treatment strategy has already demonstrated positive effects in inflammatory bowel disease (IBD) (Mottet et al. 2003). With the identification of specific HDACs important for the suppression of autoimmune- and inflammatory conditions, it will be desirable to have inhibitors specific for the exact HDAC involved at the disposition as previously discussed for applications in cancer treatment (Kristensen et al. 2009). It is not only the use of HDAC inhibitors that has been considered for the treatment of autoimmune

diseases, but also the exact opposite treatment strategy has been proposed using HAT inhibitors. HATi's have been proposed for treatment of the autoimmune disease IBD as global H4 acetylation levels normally associated with active gene expression were up-regulated in this disease (Tsaprouni et al. 2011). A comprehensive mapping of the different histone modifications in different relevant cell types is required to fully evaluate the extent to which these promising drugs can be used in the future for the treatment of autoimmune diseases.

20.5 Conclusions

Although the analysis of the genetic component of autoimmune disease has made rapid progress in the last years, the underlying etiology of autoimmune diseases is still poorly understood. We are still far from knowing the exact mechanisms underlying the different diseases phenotypes. It is not yet elucidated which environmental agents contribute to disease development and how they can induce epigenetic and transcriptional deregulation which then translates in inappropriate response of the immune system. Systematic, large scale epigenome projects such as the National Institute of Health Roadmap project in the US or the European Union initiated Human Epigenome Project (HEP) with the latter focusing on different cell types found in human blood will provide new insights into the epigenetic mechanisms. Further the HEP will provide insights in how the response to certain environmental agents will translate into molecular alterations and deregulation of gene expression that will lead to the development of the autoimmune diseases in genetically predisposed individuals. These insights might in the future provide novel therapeutic interventions targeting some of the epigenetic components of the disease process. However, more research is urgently needed to ensure a brighter future for the affected individuals.

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Chapter 21
Epigenetic Regulation of HIV-1 Persistence and Evolving Strategies for Virus Eradication

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Abstract Despite the intense effort put by researchers globally to understand Human Immunodeficiency Virus (HIV-1) pathogenesis since its discovery 30 years ago, the acquired knowledge till date is not good enough to eradicate HIV-1 from an infected individual. HIV-1 infects cells of the human immune system and integrates into the host cell genome thereby leading to persistent infection in these cells. Based on the activation status of the cells, the infection could be productive or result in latent infection. The current regimen used to treat HIV-1 infection in an AIDS patient includes combination of antiretroviral drugs called Highly Active Anti-Retroviral Therapy (HAART). A major challenge for the success of HAART has been these latent reservoirs of HIV which remain hidden and pose major hurdle for the eradication of virus. Combination of HAART therapy with simultaneous activation of latent reservoirs of HIV-1 seems to be the future of anti-retroviral therapy; however, this will require a much better understanding of the mechanisms and regulation of HIV-1 latency. In this chapter, we have tried to elaborate on HIV-1 latency, highlighting the strategies employed by the virus to ensure persistence in the host with specific focus on epigenetic regulation of latency. A complete understanding of HIV-1 latency will be extremely essential for ultimate eradication of HIV-1 from the human host.

21.1 Introduction

Viruses are obligate parasites that cannot survive without the host and infect host cells to make more progeny viruses. Some viruses like influenza and rhinovirus acutely infect the target cell to make more viruses resulting in rapid onset of the

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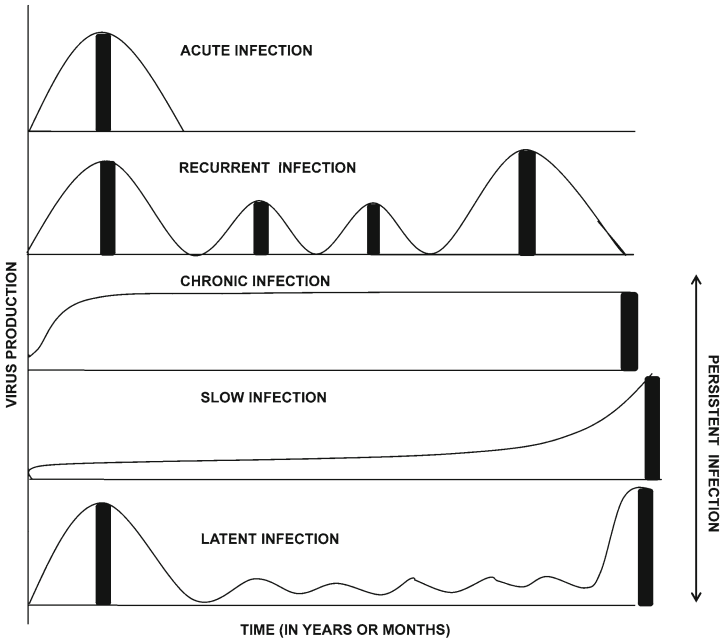

Fig. 21.1 Schematic representation of different types of viral infection and the peak of viral load with reference to time. Two types of viral infection are generally observed: Acute and Persistent infection. Recurrent infection occurs in multiple episodes of acute infection one after the other because of incapability of the immune system to completely clear the pathogen. Persistent infection can be further classified as chronic, slow or latent infection. The black bars represent the peak of the infection in a particular episode of infection

disease and clearance of the pathogen. On the other hand, persistent infections are those in which virus escapes the host's immune surveillance and is not cleared by the immune system. Persistent infections are further classified as chronic, slow and latent infections. Chronic infection is characterized by the continuous presence of the infectious virus following primary infection or recurrence. Prolonged incubation period followed by progressive disease is a hallmark of slow virus infection as seen in measles virus infection. Lack of demonstrable virus between episodes of recurrent disease is indicative of latent infection (Boldogh et al. 1996) (Fig. 21.1). Different viruses employ different strategies to ensure persistence in the host yet commonality exists between them. These strategies include (i) selection of long lived cell populations as reservoirs of virus; (ii) modulation of viral gene expression; (iii) modulation of cellular apoptotic pathway and (iv) evasion of the host immune system. Being a lentivirus, Human Immunodeficiency virus-1 (HIV-1) infects cells of the human immune system and integrates in to the host cell genome thereby leading to persistent infection in these cells. Based on the activation status of the cells, the infection could be productive or could result in latent infection. To combat HIV infection, presently used Highly Active Anti-Retroviral Therapy (HAART) uses a combination of antiviral drugs that usually suppress viremia to levels below the detectable limit

by conventional assays, i.e. 50 copies of viral RNA per ml. Cessation of HAART therapy results in viral reappearance in the circulation within a short time due to the persistence of latently infected cellular reservoirs, which has received renewed attention in recent times owing to failure of anti-retroviral therapy to eradicate HIV completely. These latently infected cells are transcriptionally silent or have low level of transcription and are permanent source for virus reactivation after discontinuation of HAART. The present chapter intends to put together our present understanding of the mechanism of HIV persistence in host cells with specific emphasis on epigenetic factors and to examine the evolving strategies to eradicate the virus.

21.2 Strategies for Viral Persistence

Viruses need reservoirs for persistence within the host. Primary requisite of these reservoirs is that they should be long lived. Herpes simplex virus (HSV), for example, uses sensory neurons as sanctuary since they are long lived and terminally differentiated cells and they serve as excellent sites for HSV persistence (Efstathiou and Preston 2005). Another good home for the viruses can be memory cells. Human immunodeficiency virus-1 (HIV-1) for example utilizes resting CD4+ T cells as reservoir (Chun et al. 1995). Stem cells being biologically immortal are sites of infinite storage for murine leukemia virus (Rosenberg and Jolicoeur 1997; Ruddle et al. 1976). Viruses also interplay with the host chromatin for its persistence. The best example to explain this would be Herpes simplex virus. HSV life cycle involves both lytic and latent infection in the host. Lytic infection takes place in the epithelial cells and sensory neurons are sites of latent infection. Latency associated transcript (LAT) is expressed in high amounts to shut off lytic gene expression. Thus viral proteins tend to regulate the latent or lytic infection. Modulation of cellular apoptotic pathways is another strategy used by viruses for persistence. For example, although HIV-1 is known to induce apoptosis in effector cells of immune system like HIV specific CTLs, but it prevents apoptosis in infected cells to allow the production of new pool of viruses from these cells. Inhibition of apoptosis by using viral proteins like Nef, Tat and gp120 or targeting some cellular factors is one of the most common strategies employed by the virus to increase the survival of these infected cells.

HIV-1 Nef has been shown to have dual effect on cellular apoptotic pathways. During the early phase of infection, it is shown to induce apoptosis of bystander cells such as CTLs by increasing the expression of FasL on the surface of infected T cells. Killing of these CTLs helps the virus to escape the host immune response (Geleziunas et al. 2001). However, during the late stage of infection, Nef inhibits apoptosis to increase the survival of HIV-1 infected cells like monocyte derived macrophages (MDMs). It also binds to p53 to protect the infected cells from p53 induced apoptosis (Greenway et al. 2002). Like Nef, Tat and gp120 are also known to modulate cellular apoptosis (Chugh et al. 2007; Swingler et al. 2007). Like viral proteins, some of the host factors also suppress apoptosis in infected cells to

promote viral persistence. CTIP2 is one such protein which increases the HIV-1 persistence in microglial cells by functioning as an anti-apoptotic factor (Cherrier et al. 2009; Giri et al. 2009). It also counteracts apoptotic function of Vpr protein. NFκB activity is also induced upon HIV-1 infection and this active NFκB appears to have a role in inhibiting apoptosis in MDMs which helps to maintain latent infection in these cells (Asin et al. 1999; McElhinny et al. 1995). TNFα production is also increased during HIV-1 infection which in turn enhances expression of Bcl-XL and Bcl-2 pro-survival proteins. These proteins further contribute in the apoptosis inhibition (Guillemard et al. 2004). Recently, role of viral RNA have been also identified in modulation of apoptotic events in infected cells (Bennasser et al. 2006; Klase et al. 2009).

Host immune evasion is another strategy used by the viruses to establish persistent infection. Virus infected cells are normally eliminated by the host immune system. Immune system recognise the viral peptides presented by MHC-I molecules on the surface of infected cells and mediate their killing by virus specific CTLs. However, in case of persistent infection, virus tries to hide from the immune system by altering the expression of these surface molecules and escape the recognition of viral antigens by immune system. This job is generally performed by those viral proteins which express early in the virus life cycle and ensure the availability of an environment that is secured from host immune response and thus suitable for establishment of persistent viral infection. In case of HIV-1 infection, Nef decreases the expression of MHC-I from the cell surface and can prevent the recognition by CTL (Schwartz et al. 1996). Tat has been also shown to inhibit the expression of MHCI molecule on the cell surface by its ability to bind TAFII250 (TATA binding protein-associated factor 250), which inhibits cellular HAT activity (Weissman et al. 1998).

21.3 HIV-1 Latency

HIV-1 is capable of infecting a range of cell types, including T lymphocytes, monocytes, macrophages, microglial cells, astrocytes, neurons, microvascular endothelial cells, etc. The virus life cycle involves various steps like virus attachment to the host cell, virus-cell fusion, uncoating, reverse transcription, integration, transcription, translation, assembly, budding and maturation. Following reverse transcription of the HIV-1 genome, linear and circular molecules of proviral DNA containing either one or two copies of the LTR are produced and accumulated as non-integrating by-products within the host cell. Only linear double stranded cDNA integrates into the host cell chromosomes. The HIV provirus can integrate into many different chromosomal locations in the cell and most infected cells harbour more than one provirus. After integration, the LTR-flanked provirus behaves as a cellular gene: the 5′ LTR operates like any eukaryotic promoter (Fig. 21.2) and the 3′ LTR acts as the polyadenylation and termination site. Integration can lead to either latent (transcriptionally inactive) or transcriptionally active forms of infection. The chromosomal milieu encountered by the provirus (integrated virus) likely helps shaping

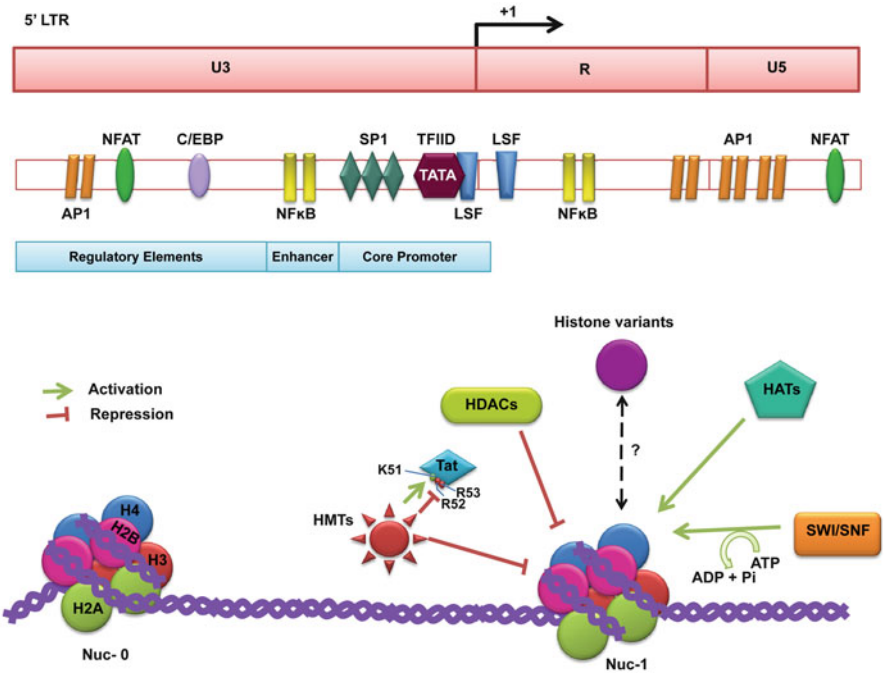

Fig. 21.2 HIV-1 long terminal repeat promoter and epigenetic factors modulating LTR driven transcription. HIV-1 LTR can be subdivided into three regions: U3, R and U5. U3 comprises of the regulatory elements, NFκB enhancer and the core promoter. Irrespective of the site of integration, LTR organizes into two distinct nucleosomes, namely, Nuc-0 and Nuc-1, with fixed relative positions of the regulatory elements. Nuc-1 displacement is necessary for transcription to proceed. Chromatin modifiers like histone acetyl transferases (HAT) and histone demethylases or remodellers like SWI/SNF participate to displace Nuc-1. In their absence, HIV transcription is repressed as is seen in the latent HIV infection. Histone deacetylases (HDACs) and Histone methyl transferases (HMTs) help establish latency in a cell by suppressing the opening of the chromatin at Nuc-1. HMTs and HATs not only function at the Nuc-1 but also on Tat protein to repress or activate Tat function. Lysine 51 methylation, for example, activates Tat's transactivation ability while arginine 52 and 53 methylation suppresses Tat activity resulting in latent situation. The role of histone variants in establishment of HIV latency remains to be elucidated

its transcriptional activity. HIV-1 integration results in proviral genome being packaged into the chromatin. The fundamental unit of chromatin is the nucleosome core/histone octamer comprising of two molecules of each canonical or core histones H2A, H2B, H3, H4 around which 146 bp of DNA wraps around. H1 is a linker histone protein that binds to the nucleosomes and the DNA wrapped around them. Nucleosome is not a rigid entity and its flexibility is needed for the opening of the chromatin and hence transcription. Irrespective of the integration site, HIV-1 LTR incorporates in two distinct nucleosomes, termed Nuc-0 and Nuc-1 with precise location of the regulatory elements as depicted in Fig. 21.2. Remodelling (altering the histone-DNA interaction) at Nuc-1 is needed for successful transcription of the

HIV genome. Chromatin remodelers and modifiers function at Nuc-1 to open the chromatin for transcription to proceed (Fig. 21.2). This aspect will be discussed in detail later in the chapter.

Resting memory CD4+ T-cells are a well-defined latent reservoir of HIV. The reservoir of resting memory $CD4^+$ T cells is established during primary infection. *In vivo* presence of HIV-1 latent reservoir was first time shown in 1995 by Chun and coworkers (Chun et al. 1995), when integrated proviruses were seen in purified resting $CD4^+$ cells from HIV-1 patients. These cells pose various blocks at the transcriptional and post transcriptional level leading to the persistence of transcriptionally silent viral genome in the host cell. During antiretroviral therapy, these latent cells die very slowly with an average half-life of 44 months, indicating that under current therapeutic regimen it will take over 60 years to deplete this reservoir. Despite intense research, the molecular mechanism of HIV-1 latency remains incompletely understood. Thus, it is necessary to unravel the molecular mechanism regulating viral latency, which may lead to development of novel strategies aimed at latent stage of virus and thus complete eradication of HIV.

21.3.1 Types of Latency

21.3.1.1 Pre-integration Latency

This is a stage of virus life cycle represented by HIV infected resting CD4+ T cells which carry partially reverse transcribed unintegrated viral genome in their cytoplasm (Zack et al. 1990). Lower levels of ATP in these resting cells (Korin and Zack 1999; Meyerhans et al. 1994) not only slows down reverse transcription of viral RNA but is also incapable to support ATP dependent nuclear import of large HIV-1 pre-integration complex (Bukrinsky et al. 1992). Hyper-mutation of viral DNA by host restriction factor APOBEC3 restricts viral replication in resting $CD4^+$ T cells (Chiu et al. 2005) and is therefore thought as one of the mechanism for induction of pre-integration latency. It represents a short lived form of viral latency which is commonly seen in untreated individuals. Due to this labile nature, it has gained less clinical concern than the stable form of latency discussed below.

21.3.1.2 Post-integration Latency

It represents a non-productive state of viral infection commonly seen in resting $CD4^+$ memory T lymphocytes carrying a transcriptionally dormant provirus integrated into their genome. It is one of the major regulators of viral persistence in HIV-1 patients receiving HAART therapy (Chun et al. 2003) and is an extremely stable form of latency with a half life of about 44 weeks. This transcriptional dormancy of viral genome can be explained at the molecular level by several different mechanisms:

i. **Nature of integration site:** Chromosomal positioning of a gene has been shown to be an important regulator of gene expression. Initial *in vitro* studies in infected T lymphocytes (Jordan et al. 2003) have shown that viral genome preferentially integrates in the repetitive DNA elements of host genome heterochromatic region. However, some later studies in human T lymphocytic cell lines (Lewinski et al. 2006; Schroder et al. 2002) and in resting CD4+ T cells from patients under HAART therapy (Han et al. 2004), have reported integration of proviral DNA in the intronic regions of actively transcribing gene. All these studies indicate that site of integration in host genome has an important role in regulation of viral latency.

ii. **Transcriptional Interference (TI):** Transcriptional interference is an inhibitory effect of one transcriptional process on a second transcriptional process (Adhya and Gottesman 1982). Integration of viral genome downstream to a highly active host gene promoter can create TI situation leading to latency. In such a situation, the elongating RNA polymerase reads through the downstream promoter preventing its transcription initiation. Thus HIV-1 genome that integrates in the introns of active genes (Han et al. 2004) is transcribed as part of the intron in TI situation and is eventually spliced out and degraded (Lenasi et al. 2008). Recent reports indicate the involvement of chromatin reassembly factors (CRFs) in the transcriptional interference (Gallastegui et al. 2011).

iii. **Differential expression of Transcriptional activators:** HIV-1 gene expression is under the regulation of various cellular factors which has their binding sites on viral LTR like NFκB, NFAT or Sp1 (Nabel and Baltimore 1987). Enhancer region of HIV-1 LTR is also known to respond to T cell activation signals like IL2, TNFα, etc. (Bohnlein et al. 1988; Duh et al. 1989; Tong-Starksen et al. 1987). Differential expression of these transcription stimulators and their limited availability in resting CD4+ T lymphocytes contributes in the maintenance of latency. Murr1, a host restriction factor has been shown to inhibit HIV-1 replication in resting CD4+ T cells by inhibiting NFκB activity and thus contributes in maintenance of HIV-1 latency in quiescent T cells (Ganesh et al. 2003). Resting T cells also show reduced expression of CDK9 and CyclinT1, the two essential components of P-TEFb, and thus pose a barrier to viral transcription (Garriga et al. 1998; Herrmann et al. 1998; Sung and Rice 2006).

iv. **Availability of viral factors like Tat and Rev:** HIV-1 transcription initiates from the LTR promoter and short transcript of approximately 60 bases called TAR (transactivation response element) is made that creates a binding site for Tat. Tat binds to TAR and then recruits pTEFb kinase complex on HIV-1 LTR to promote the phosphorylation of C-terminal domain (CTD) of RNA Pol II (Zhou et al. 2000) and thereby enhancing the production of viral transcripts. Tat is also known to interact with various cellular factors like elongation factor ELL2 (He et al. 2010), acetyltransferase p300/CBP (Benkirane et al. 1998; Bres et al. 2002; Kiernan et al. 1999), ATP dependent chromatin remodelling complex SWI/SNF (Mahmoudi et al. 2006), which either modify Tat or recruit transcriptional activator molecules to induce HIV-1 LTR mediated viral transcription. This predominant role of Tat in HIV-1 transcription suggests that the

absence of Tat or its mutation can result in inefficient transcription leading to HIV-1 latency as is evident by relatively low levels of viral transcription observed in case of latently infected U1 and ACH2 cell lines carrying mutation in Tat and TAR region respectively.

Rev protein is involved in export of viral transcripts from nucleus. A threshold level of Rev expression is required to rescue the unspliced 9.2 kb viral transcript from the nucleus (Pomerantz et al. 1990, 1992). However, the absence of this can lead to latency as is seen in relatively miniscule levels of unspliced 9.2 kb transcript than the singly spliced 4.3 kb or the multiply spliced 2 kb transcripts in latent cell lines (Pomerantz et al. 1992). In addition to Tat and Rev, viral proteins Nef and Vpr have been also proposed to play some role in HIV-1 latency (Levy et al. 1995; Niederman et al. 1989).

21.4 Epigenetic Regulation of HIV Latency

The term "Epigenetic" means a change in the gene expression that does not involve changes in the gene sequence. HIV-1 utilizes a number of epigenetic strategies to regulate latency, the important ones of which are discussed below:

21.4.1 Post-translational Modifications

Various disparate post-translational modifications (acetylation, methylation, ubiquitination, sumoylation, ADP-ribosylation, phosphorylation) http://www.sciencedirect.com/science/journal/0959437X of the proteins called histones that package the DNA to form the "chromatin" play an important role in epigenetic regulation (Berger 2002). In the following sections we will discuss each of these epigenetic modifications in case of HIV-1 infection (Fig. 21.3):

i. **Acetylation and deacetylation**
Acetylation and deacetylation are known to regulate the accessibility of the transcription complex to the chromatin. Acetylation is associated with opening of the chromatin so that transcription factors can gain access to it. Deacetylation, on the other hand, makes the chromatin inaccessible. Best studied post-translational modification in case of HIV is acetylation-deacetylation of histones. Key participants in this strategy are Histone deacetylases (HDAC) and Histone acetyl transferases (HAT). Four classes of HDACs have been reported in the literature, namely, Class I, Class II, sirtuins and class IV (de Ruijter et al. 2003). HDAC 1, 2, 3 and 8 fall in the category of class I HDACs that are related to the yeast RPD3 HDAC. HDAC 4, 5, 6, 7, 9 and 10 form the class II HDACs which are related to the yeast HDA1 HDAC (Verdin et al. 2003). Sirtuins are Sir2-related NAD^+

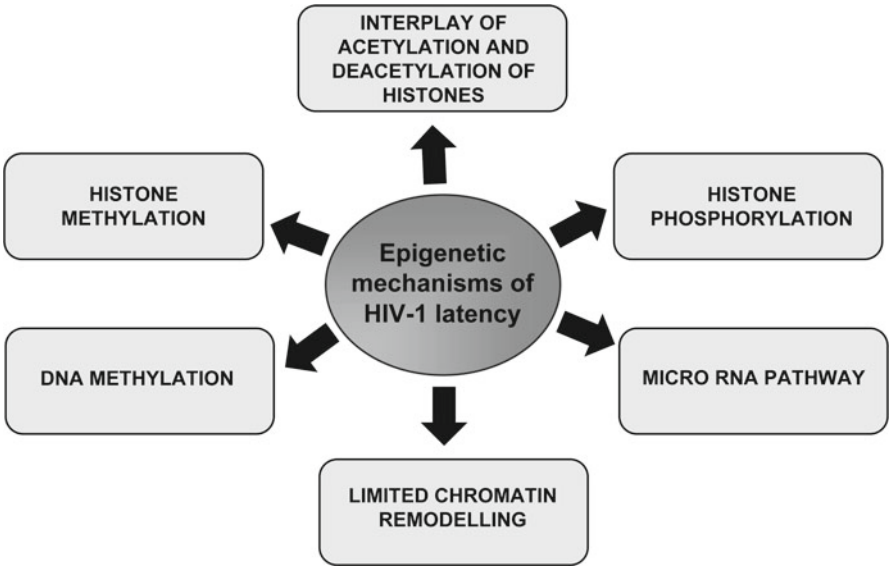

Fig. 21.3 Schematic representation of different epigenetic mechanisms leading to HIV-1 latency. Different mechanisms are responsible for maintenance of HIV-1 latency. Epigenetic mechanisms that function to maintain HIV-1 latency involves post translational modifications of histones like interplay of histone deacetylation and acetylation, histone methylation and phosphorylation. Other epigenetic mechanisms which are contributors of latency are DNA methylation, micro RNA pathway and chromatin remodelling. Absence of the chromatin remodelers limit nucleosome flexilibility to remodel, thus restricting active transcription and leading to HIV-1 latency

dependent deacetylases that form the third class of HDACs. Members in this class include SIRT 1–7. HDAC11 shows similarity with class I HDACs but similarity is not very significant so it is a unique member of class IV HDACs (Gao et al. 2002; North and Verdin 2004).

Histone deacetylase HDAC1 has been shown to be recruited by wide array of cellular factors like YY1, LSF, c-Myc, SP1, CTIP2, CBF1, NFκB p50- at the integrated LTR promoter (Coull et al. 2000; Imai and Okamoto 2006; Jiang et al. 2007; Marban et al. 2007; Tyagi and Karn 2007; Williams et al. 2006). A study was performed to look for the specific HDACs which could be plausible contributors to latency. Chromatin immunoprecipitation studies showed that HDAC2 and HDAC3 were recruited at the LTR promoter and have repressive activity (Keedy et al. 2009).

Histone acetyl transferase (HAT) family includes p300/CBP, PCAF (p300/CBP Associating Factor), Tip 60, TAFII250 and GCN5. Although these HATs play an important role in cellular transcription but HIV also exploits these proteins for transcription from the LTR promoter. Tat engages all the five HATs to establish the virus in to the host cell. Tat interacts with and recruits p300/CBP to the LTR promoter (Marzio et al. 1998; Wong et al. 2005) where it acts as coactivator of Tat. Tat actually forms a ternary complex with p300 and PCAF and

increases its affinity for PTEFb/CDK9 complex (Benkirane et al. 1998; Deng et al. 2001). Apart from its histone acetylation activity, p300 acetylates viral integrase and Tat. This acetylation at Lys 50 and Lys 51 of Tat is essential for its activity (Ott et al. 1999). Acetylated integrase regulates integration. Vpr also requires help of p300 for its transcriptional activation activity. Thus p300 participates at multiple stages of HIV-1 life cycle. PCAF is another HAT that participates extensively in HIV life cycle. PCAF interacts with Lys 50 acetylated Tat to serve as a coactivator of HIV transcription. Like p300, PCAF also activates transcription from LTR promoter by acting in synergy with Tat protein (Bres et al. 2002; Dorr et al. 2002). GCN5 also acetylates Tat at same residues K50 and K51 as p300 and mediating same function of LTR activation (Col et al. 2001). Tat also interacts with $TAF_{II}250$ (Weissman et al. 2001) to repress transcription from $TAF_{II}250$ dependent MHCI genes (Weissman et al. 1998). Tip60, a HAT originally identified as a Tat interacting cellular protein also assists Tat in transactivation of the LTR promoter (Kamine et al. 1996). Looking at the details of HIV-1 LTR driven gene expression, it becomes obvious that it is a concerted action of many host and viral proteins that brings about successful transcription. A cell can house latent reservoirs and stay dormant lifelong because of the absence of any of these critical factors indispensable for HIV transcription.

ii. **Methylation**

Another post translational modification of histones gaining relevance in HIV biology is methylation. Genomic features decide the integration sites for HIV genome. HIV avoids integration in regions with transcription inhibitory modifications such as H3K27 and DNA CpG methylation (Wang et al. 2007). A recent report suggests that chromatin mediated repression is also mediated by Suv39H1, heterochromatin protein HP1γ and histone H3K9 trimethylation. Histone H3 is tri-methylated at lysine 9 by Suv39H1, a H3K9 methyl transferase (Rea et al. 2000). HP1 (heterochromatic protein 1) is found to be associated with Suv39H1 (Aagaard et al. 1999) and binds to regions methylated by it (Bannister et al. 2001) recruiting more Suv39H1 leading to maintenance of hypermethylation and the chromatin repressive state (Grewal and Moazed 2003; Maison and Almouzni 2004).

Studies performed with *in vitro* cellular models and *in vivo* in PBMCs from HIV-1 infected donors have shown that reactivation can be mediated by silencing of HP1γ (du Chene et al. 2007). Latent proviruses are observed to have heterochromatic markers at the viral LTR. Restrictive chromatin structures at the viral LTR drag HIV into latency (Pearson et al. 2008). Evidences exist in literature about the role of H3 lysine 9 (H3K9) methyltransferase G9a in mediating repressive dimethylation at H3K9 at the LTR promoter. Studies show that G9a inhibitor BIX01294 reactivates expression of HIV-1 from cellular models of latency, ACH2 and OM10.1 (Imai et al. 2010). It is not only methylation of histones that participates in HIV life cycle but methylation of Tat also plays a crucial role for its functional activity. Tat methylated on R52 and 53 by PRMT6 decreases its association with transcription factors and the result is reduced transcription from the LTR (Boulanger et al. 2005; Xie et al. 2007). Tat methylated on K51 by H3-K9 methyl transferase SETDB1 also inhibits its

function and hence reduces transcription from the LTR (Van Duyne et al. 2008). Contrary to this, K51 methylation by Set7/9-KMT7 increases Tat activity on the LTR (Pagans et al. 2010). This clearly indicates that specific methylation inhibitors need to be evaluated for their ability to activate the latent reservoirs. Such methylation inhibitors should not be inhibitory to Tat's function leading to latency but should work solely on the LTR activation purging the hidden latent reservoirs.

iii. **Phosphorylation**
Phosphorylation of Histone H1 by PTEFb at the S/TPXK sequence is known to be important in regulating its chromatin binding. This phosphorylation takes place in a Tat specific manner (O'Brien et al. 2010). Mutant analysis of H1 has shown that in absence of this phosphorylation, Tat transactivation is inhibited. Absence of this phosphorylation depletes the H1 mobility and HIV-1 transcription, giving birth to latent reservoir of HIV in these infected cells.

21.4.2 Chromatin Remodelling

Chromatin-modifying complexes can be classified in two groups: first group includes factors that mediate covalent modification of histones. The protruding N-terminal tails of the histone proteins in the nucleosome are substrates of various post translational modifications like methylation, acetylation and phosphorylation. Histone acetyl transferases like GCN5, p300/CBP, PCAF are included in this family. The second group includes proteins that change the location or conformation of nucleosomes utilizing the energy from the hydrolysis of ATP and thus increasing the accessibility to the DNA (Hargreaves and Crabtree 2011). SWI/SNF is an ATP-dependent chromatin remodelling complex belonging to this class that acts to reorganize chromatin structure so as to facilitate binding of transcription factors (Kwon et al. 1994). Integrase interacting protein (Ini1 or SNF5), BRM and BRG1 are the core subunits of SWI/SNF complex, which participate in expression of many eukaryotic genes. Nuc-1 at the LTR requires remodelling via SWI/SNF to initiate transcription. Ini1 and BRG1 interact with Tat to recruit SWI/SNF remodelling complex at Nuc-1 and to activate the transcription from the LTR promoter (Bukrinsky 2006; Mahmoudi et al. 2006; Treand et al. 2006). Inactive or lower levels of SWI/SNF complex in a cell limit this remodelling at Nuc-1 leading to absence of long HIV transcripts and eventually progressing to latency.

21.4.3 Histone Variant Proteins Forming the Nucleosomes

Histone variants (reviewed extensively in Henikoff et al. 2004) for H2A and H3 are being studied since decades for their role in epigenetic regulation. These variants differ in few amino acids. As for example, H3, the core histone and H3.3, the H3 replacement variant vary by four amino acids only. H3.3 replaces H3 soon

after the initiation of transcription (Ahmad and Henikoff 2002; Janicki et al. 2004). H2A.Z is a variant associated with active chromatin. Specific protein assembly complexes regulate the exchange of the core histones with the variants in the nucleosome. SWR1 and HIRA complex deposit H2A.Z and H3.3 respectively. SWR1 complex destabilizes the nucleosome, exchanging H2A.Z-H2B for H2A-H2B. CENP-A is yet another H3 histone variant found at the mammalian centromeres (Palmer et al. 1991). Some variants differentiate between the active and the silenced chromatin like the MacroH2A which is enriched on the human inactive X chromosome. Another variant H2A-Bbd, however, is deficient in the inactive X chromosome. Nucleosomes containing H3.3/H2A.Z double variant marks the nucleosome free regions of the active promoters, enhancers and insulators (Jin et al. 2009). Histone variants have elaborate roles in gammaherpesvirus latency and HSV-1 lytic infection, but this area remains less explored in HIV transcription and latency.

21.4.4 Methylation of DNA

DNA methylation also extends repressive effect on the transcription from the LTR (Blazkova et al. 2009; Kauder et al. 2009). HIV-1 encodes for two CpG islands that are seen to be methylated during latency. This methylation recruits transcriptional repressors like MBD2 (methyl CpG binding domain family of proteins) to the HIV-LTR. Latent HIV-1 has been observed to be reactivated by the use of aza-CdR, thereby depicting the role of cytosine methylation in case of HIV latency (Blazkova et al. 2009; Kauder et al. 2009). There are increasing evidences in literature regarding role of histone and CpG methylation in the maintenance of HIV latency.

21.4.5 Micro RNA

Micro RNAs (miRNAs) are 22–25 nucleotide long, endogenous, non coding RNA that have many regulatory functions. miRNAs bind with imperfect complementarity to their targets (3′ UTR) and repress the translation of the mRNA which is eventually degraded in the P bodies. Resting T cells that harbour latent HIV-1 provirus are flooded with miRNAs that have predicted binding sites in the HIV-1 genome (Huang et al. 2007). Many host miRNA like miR-28, 125b, 150, 223 and 382 have been reported to be inducers of HIV-1 latency. To circumvent restrictions on their replication by miRNA, viruses use RNA silencing suppressor (RSS). A similar mechanism also exists for HIV where it fights against the killing strategies of the host. Dicer and Drosha, the two key participants of the miRNA processing machinery were found to be inhibitory to the HIV replication both in PBMC from the

infected individuals and latently infected U1 cells. HIV, in turn, suppresses the expression of miRNA cluster miR-17/92 to overcome these hindrances for its own survival (Triboulet et al. 2007). Many such see-saw relationships exist in case of HIV. Some of these host proteins and miRNAs are directed to suppress HIV-1 production but HIV-1 is intelligent enough to exploit these factors for its own benefit. Host miR-29a functions to increase the interaction of HIV-1 mRNA with RCK/p54, a P-body protein (Nathans et al. 2009). P-bodies (processing bodies) are cytoplasmic foci that participate in mRNA degradation and translational repression. They contain factors for mRNA turnover like mRNA degradation machinery, decapping enzymes etc. (Parker and Sheth 2007). Thus, host miR-29a-HIV-1 mRNA interaction plunges HIV-1 mRNA to P-bodies possibly targeting it for translational suppression or its decay as is evident by increased virus production upon depletion of the P-bodies or RCK/p54. Alternatively, the virus could be utilizing this interaction to suppress translation of its mRNA and hide itself till favourable conditions reappear (Nathans et al. 2009).

To prepare the cellular milieu conducive for its survival, HIV-1 changes the profile of the miRNAs (Yeung et al. 2005), utilizing key regulatory protein of the RNA silencing pathway, TRBP, for transcription of TAR containing transcripts (Haase et al. 2005). HIV-1 TAR RNA sequesters TRBP, thus suppressing the host RNA silencing machinery (Bennasser et al. 2006). Virus also encodes for miRNA, named "vmiRNA", that functions to fight against the strategies employed by the host (Bennasser et al. 2004; Ouellet et al. 2008). vmiRNA are also suspected to contribute to latency. HIV-1 TAR RNA is processed by Dicer to produce a vmiRNA that recruits HDAC1 to the LTR indicating its role in viral latency (Klase et al. 2007).

Cellular anti HIV-miRNAs restrict HIV-1 to flourish in the monocytes where they are found in abundance (Wang et al. 2009). They are also major contributors of latency (Huang et al. 2007). Anti-miRNA inhibitors are possible therapeutic candidates that can be used to induce these latent reservoirs for clearance by HAART (Zhang 2009). All the above epigenetic contributors to HIV-1 latency have been diagrammatically presented in Fig. 21.3.

21.5 Experimental Models of HIV-1 Latency

Previous studies on HIV-1 latency have depicted the role of various cellular and viral factors in maintaining latency which is suggestive of a multifactorial mechanism behind it. Relative low frequency of these cells in HIV patients and the absence of distinct latency markers which can distinguish between resting $CD4^+$ T cells and the latently infected cells, limits the use of various experimental approaches to study mechanism of latency directly from primary cells. To circumvent this problem, various model systems have been utilized to study HIV-1 latency using chronically infected primary cells or cell lines and animal models. Different *in vitro* and *in vivo* cellular models of HIV-1 latency are listed in Table 21.1.

Table 21.1 Various model system for studying HIV latency

S.No.	Model system	Host	References
1	ACH2	A3.01 CD4+ T cell	Folks et al. (1989)
2	JΔK	Jurkat CD4+ T cell	Antoni et al. (1994)
3	U1	U937 promonocytic cell	Folks et al. (1987)
4	J Lat	Jurkat CD4+ T cell	Jordan et al. (2003)
5	OM10.1	HL60 premyelocytic cell	Butera et al. (1991a, b)
6	SCID-hu (Thy/Liv)	Scid/scid mouse	Brooks et al. (2009)
7	SIV-macaque	macaque	Hazuda et al. (2004)
8	Hu-Rag$^{-/-}$gamma(c)$^{-/-}$	mouse	Choudhary et al. (2009)

21.5.1 Cell Lines as Latency Model System

Various cellular model systems have been developed which resemble with HIV-1 latent state using chronically infected cell lines and clones. These model cell lines normally show very low level of viral gene expression however they can be activated by activator molecules like cytokines, PMA, TNFα, etc., to produce enhanced levels of viral proteins.

U1 (Folks et al. 1987) is a chronically infected clone derived from promonocytic cell line U937 (Pomerantz et al. 1990). It carries two copies of non replicating HIV-1 provirus. Latency in this cell line is a result of reduced activity of Tat which occurs due to mutations in both the alleles of Tat. One of the tat allele lacks initiation codon while the other allele carries an H to L mutation in amino acid 13 (H13L) (Emiliani et al. 1998). ACH2 is also a chronically infected clone derived from A3.01 cell line and carries one copy of HIV-1 provirus (Folks et al. 1989). Latency is established in this clone due to a point mutation in TAR which in turn results in impaired HIV-1 Tat function (Emiliani et al. 1996). JΔK is another chronically infected clone derived from Jurkat cell line (Antoni et al. 1994). Latency is characterized in this clone due to the deletion of NFκB sites from the HIV-1 LTR. J-Lat model was generated from Jurkat cell line infected with a retroviral vector carrying the whole HIV-1 genome lacking the Tat gene. It carries a GFP ORF under the HIV-1 LTR promoter (Jordan et al. 2003). OM 10.1 cells are clonally derived from HL60 premyelocytic cell line that harbors a single latent HIV-1 provirus (Butera et al. 1991a, b).

Latent cellular models have been used to understand the role of histone methylation in HIV-1 latency. BIX01294, an inhibitor of H3K9 methyl transferase, G9a has been shown to activate HIV-1 latent reservoirs from ACH2 and OM10.1 cells (Imai et al. 2010). U1 cells have been used to understand the role of HP1γ in chromatin mediated repression of LTR promoter and HIV latency (Isaure du Chene; EMBO J 424). HIV-1 proviral latency is maintained in J-Lat cells through local histone deacetylation on HIV-1 LTR mediated by NFκB1-p50-HDAC1 complex binding and thus repressing the active transcription (Williams et al. 2006). These cell lines have extensively been used in the HDAC class I and II inhibitor studies (Ylisastigui et al. 2004; Archin et al. 2009; Matalon et al. 2011). J-Lat cells have also been used to understand the role of DNA methylation in maintenance of HIV-1 latency.

LTR promoter is hypermethylated in J Lat cells, MBD2 being the key player binds to the two CpG islands near the transcription start site, maintaining the transcriptionally repressive latent state (Kauder et al. 2009). DNA methylation inhibitor studies in J-Lat cells have experimentally shown to reactivate latent HIV.

Mechanistic studies with these model cell lines revealed the critical role of site of integration in viral persistence. In these latent cell lines, integration was majorly seen in the heterochromatic region of genome (Jordan et al. 2003), however, when similar studies were done with resting CD4+ T cells from HIV-1 infected patients undergoing HAART therapy (Liu et al. 2006) or untreated HIV-1 infected PBMC (MacNeil et al. 2006), the preferred occupancy for proviral integration was found in transcriptionally active genes as reviewed earlier in this chapter. This contrasting picture of proviral integration between these model cell lines and the latently infected primary cells as well as the absence of Tat and TAR mutation during the *in vivo* latent infection have recently raised a concern over the usage of these cellular models to study the *in vivo* HIV-1 latency.

21.5.2 Animal Models of HIV Latency

To study the mechanistic aspects of HIV latency, Zack and colleagues developed a mouse model called SCID (Severe combined immunodeficiency)-hu (Thy/Liv) mouse model (Brooks et al. 2001). It is an immunodeficient CB17 scid/scid mouse which lacks functional T and B cells. It was transplanted with human fetal liver tissue to provide source of hematopoietic progenitor cells and thymus tissues to create a microenvironment for differentiation and proliferation of these cells. These grafted tissues were infected with HIV by injecting the virus into the graft leading to infection of $CD4^+$ thymocytes. When these infected cells undergo several rounds to replication and differentiation, some of the mature resting $CD4^+$ cells carry the latent viral genome as they show minimal HIV-1 LTR mediated transcription.

Due to the close mimicry of these cells with the *in vivo* latently infected quiescent $CD4^+$ cells, this mouse model was used to develop new strategies to eradicate viral latent reservoir. Though this model represents a suitable source of high frequency production of quiescent $CD4^+$ single positive (SP) cells, however, the predominant source of latent infection i.e. infected $CD4^+$ memory T cells are absent in this system. Furthermore, the differences in the distribution, activation threshold and TCR mediated signaling pathways (Dutton et al. 1998; Farber et al. 1997; Jenkins et al. 2001) of naive T cells and memory T cells, suggests that SCID hu Thy/Liv mouse can function as a good model for studying the HIV latency in quiescent T cells but to study the *in vivo* latency, a more efficient model system is required.

To further enhance the mimicry between the latency model system and HIV-1 infected humans, another animal model was developed using Simian immunodeficiency virus infected macaque (SIV-macaque). These SIV-macaques were treated with reverse transcriptase inhibitors to decrease the viral load to undetectable level (<100 copies/ml) (Hazuda et al. 2004). Latently infected $CD4^+$ cells

carrying integrated SIV DNA were recovered from lymph node, peripheral blood and spleen of these animals. Later on, various studies have been done in these models using various antiretroviral drugs to mimic the situation of HIV-1 infected latent cells in humans (Hazuda et al. 2004; Hofman et al. 2004). Another humanized mouse, Hu-Rag$^{-/-}$ gamma(c)$^{-/-}$ was also used to study HIV persistence (Choudhary et al. 2009). Although these models are yet to be exploited well in epigenetic studies but they seem to have future potential in unravelling epigenetic mechanisms leading to HIV-1 latency.

21.5.3 Use of Primary Cells as Latency Model

Various recent studies have utilized primary cells as model to study HIV-1 latency. Saleh and colleagues have shown that resting CD4$^+$ T cells after incubation with CCL19 and CCL21 can efficiently allow viral integration with restricted viral gene expression representing a model of post integration viral latency (Saleh et al. 2007). Another such model was developed by Yang and colleagues, where resting CD4$^+$ T cells were first transduced with a lentiviral vector coding BCL2 cDNA followed by activation with anti-CD3/CD28 and infection with HIV-1. These infected cells moved to a latent state after the removal of cytokines (Yang et al. 2009). This model was used to screen compounds that can reactivate the latent reservoir without activating the T cells globally. Another such model of HIV latency was developed by Planelles group using primary CD4$^+$ T cells. This model was used to generate latently infected resting central memory T cells (Bosque and Planelles 2011). Such primary CD4$^+$ T cell models have been employed in the study of HDAC inhibitors which have shown good potential in reactivation of latent HIV in cell line models (Sahu and Cloyd 2011; Tyagi et al. 2010). Role of heterochromatic protein HP1γ has been also implicated in maintenance of latency in PBMCs from HAART treated HIV infected individuals (du Chene et al. 2007).

21.6 Eradication of Latent Reservoirs

Latent cells are generally characterized by their resting nature and presence of a dormant virus. These cells also show high resemblance to the uninfected resting T cells and almost appear indistinguishable. This dormant nature of HIV virus in latent cells as well as their high resemblance with uninfected resting T cells poses a great hurdle to understand the molecular nature of HIV latency and to develop highly specific and effective therapeutic strategy. Current strategies aim to activate these latent cells making them more sensitive towards immune response to purge the virus by immune effector cells as well as antiviral agents. However, with the activation of latently infected cells, the threat of new rounds of infection always remains there. So, the new anti-viral therapy should include such agents which can

boost up the host immune response and specifically target the latently infected cells. Currently evolving anti-HIV therapeutic strategies involve various ways to activate these latent reservoirs and are being discussed briefly below:

21.6.1 Induction of HIV Transcription from Latent Cells

Intermittent administration of cytokines like IL-2 and IL-7 in HIV patients along with HAART therapy have shown to be effective in reducing the pool of latent cells carrying transcriptionally silent virus and thus seems to be a promising strategy for latent virus eradication (Chun et al. 1999; Scripture-Adams et al. 2002). Modulation of cellular transcription is another common strategy used to induce viral transcription. Cellular transcription is generally modulated by use of HDACs inhibitors like Valproic acid (Blankson et al. 2002; Lehrman et al. 2005), methylation inhibitors (Kauder et al. 2009) and combining these agents with HAART therapy can help to clear the viral reservoirs. It is a promising approach to purge the latent reservoirs which can be then cleared by HAART therapy. Several HDAC inhibitors are being tested for their potency in activating the silent reservoirs. Valproic acid (VPA), a drug in clinical use was tested in resting CD4 cells of aviremic HIV infected patients treated with highly active antiretroviral therapy (HAART). VPA induced acetylation at the integrated HIV promoter and also production of virus from the resting CD4 cells of infected patients on HAART (Ylisastigui et al. 2004). Potency testing was done for various inhibitors of class I and II HDACs, namely, MRK1, MRK4, MRK10, MRK11, MRK12, MRK13, MRK14, apicidin and VPA. Inhibitors of class I HDACs proved to be efficient inducers of virus production from resting CD4 cells of aviremic patients on ART by induction of LTR transcription. Currently the focus is being laid on HDAC inhibitors, already in clinical use for treatment of some other disease. Suberoylanilide hydroxamic acid or Vorinostat (SAHA) (Contreras et al. 2009), Romidepsin (Depsipeptide, FK-228), Belinostat (PXD101) and LAQ824/LBH589 are HDAC inhibitors which can be put to clinical use. Other promising epigenetic targets for anti HIV therapy are extensively reviewed in Varier and Kundu (2006).

Activation of NFκB pathway can also drive the HIV-1 LTR driven gene expression from latently infected cells. In this direction, Prostatin was found to very promising as it can increase the recruitment of active NFκB on HIV-1 LTR (Blankson et al. 2002; Williams et al. 2007) and was also shown to increase viral gene expression and decrease CD4 expression in PBMC from patients on HAART therapy (Kulkosky et al. 2001). NFκB independent activators of HIV-1 transcription were also found to be effective for purging the viral reservoirs. NFκB is a transcription factor which is present in almost all animal cell types and found to be associated with most of the signalling pathways. Therefore, activation of NFκB pathways can result in global activation of T cells. To avoid this, a hunt for transcription factors which can induce HIV-1 transcription in a NFκB independent manner has been started. Recently, Yang and co-workers have shown that resting memory T cells from patients on HAART therapy can be reactivated with Est1 transcription factor to induce virus transcription without activating T cells globally (Yang et al. 2009).

Hexamethylene bisacetamide (HMBA) is a bipolar compound, which can induce Tat independent HIV-1 transcription. It was shown recently that it can activate HIV-1 transcription in latently infected CD4+ cells isolated from aviremic patients under ART therapy by a Tat independent mechanism (Choudhary et al. 2008; Klichko et al. 2006). It seems to activate PI3K/Akt pathway to enhance the recruitment of active P-TEFb complex on HIV-1 LTR to induce viral gene expression (Contreras et al. 2007).

21.6.2 Manipulation of Host miRNA Profile

Many host miRNA which have their binding sites in the viral genome have been shown to be inducers of viral latency (Huang et al. 2007) and have been extensively discussed in an earlier section of this chapter. Manipulation of these miRNAs to prevent their binding on viral mRNA or use of anti-miRNA inhibitors seems to be a possible therapeutic strategy for plunging these latent reservoirs for clearance by HAART (Zhang 2009).

21.6.3 Use of Combinatorial Therapy

Recently, the concept of a combination therapy involving use of various combinations of HIV-1 transcription activators discussed above is gaining interest. Transcriptional activators like Valproic acid or suberoylanilide hydroxamic acid (SAHA) with prostatin have shown synergistic effect on reactivation of latent cell lines like U1 and J-Lat cells, purging the latent HIV more potently from these cells as compared to the individual inhibitor treatment. Similar effects were observed in PBMC isolated from HAART treated aviremic patients (Reuse et al. 2009). It is believed that the future therapeutic regimen development against HIV can be inspired by such combinatorial approach (Fig. 21.4) to eradicate the hidden virus more efficiently from the infected host.

21.7 Concluding Remarks

Latent reservoirs are greatest obstacle in treating HIV patients and ultimate eradication of virus. HIV eradication is a hard nut to crack until these pools are cleared from the infected individual since HAART therapy is incapable of handling and clearing these hidden reservoirs. According to our current understanding, intelligent design of antiretroviral regimen is needed to ensure complete eradication of the virus from the host and not only the active virus. Presently, HAART therapy is a triple therapy

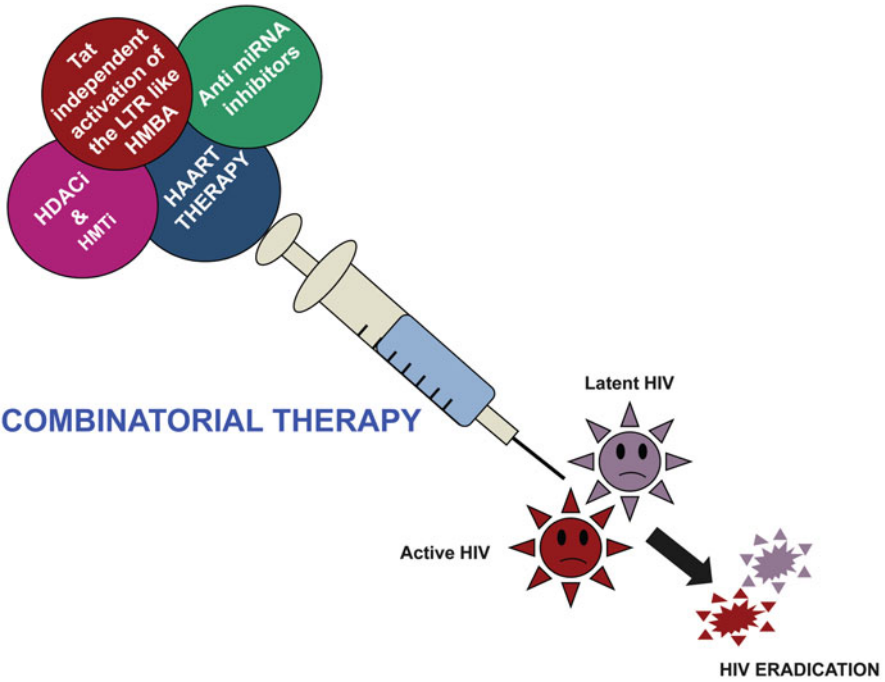

Fig. 21.4 Schematic representation of the design of a future combinatorial therapeutic regimen. HAART therapy is inactive against the latent reservoirs. To make it active for the latent reservoirs, supplementation of HAART with HDAC, HMT, anti-miRNA inhibitors is necessary. Compounds like HMBA can also be included in the combinatorial therapy for the activation of the silent promoter in Tat-independent manner. These supplements would purge the latent reservoirs on which the HAART can act and eradicate the virus

which normally includes one protease inhibitor and two nucleoside or non-nucleoside reverse transcriptase inhibitors or other such combinations. A combinatorial therapy keeping in mind activation of latent virus should be tested for clinical use. The possible design of the therapy can be present triple therapy supplemented with epigenetic silencing inhibitors like HMTi, HDACi, anti-microRNA inhibitors and compounds like HMBA. Lack of complete understanding of the molecular mechanisms operating in maintenance of latency could be another drawback that is a plausible hurdle to purge these latent reservoirs. Histone variants is one such avenue that is unexplored in case of HIV but there are increasing evidences of their roles in latency of other viruses like gammaherpesvirus and HSV-1. Thus, we need to actively work on these aspects of HIV biology to have an AIDS-free future across the globe.

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Chapter 22
Epigenetics in Parkinson's and Alzheimer's Diseases

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Abstract Neurodegenerative disorders, such as Parkinson's and Alzheimer's disease, are highly complex, due to their multifactorial origin, not only depending on genetic but also on environmental factors. Several genetic risk factors have already been associated with both the diseases, however, the precise way through which the environment contributes to neurodegeneration is still unclear.

Recently, epigenetic mechanisms, such as DNA methylation, chromatin remodeling or miRNAs, which may induce alterations in genes expression, have started to be implicated in both AD and PD. Epigenetic modulation is present since pre-natal stages and throughout lifetime, and depends on lifestyle conditions and environmental exposures, and consequently could represent the missing link between risk factors and the development of sporadic disorders. This chapter will discusses the role of epigenetics in AD and PD.

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22.1 Introduction

Neurodegenerative disorders, such as Alzheimer's disease (AD) and Parkinson's disease (PD) affect a growing number of people due to the increase in life expectancy. The world health organization estimates that by 2,025 three-quarters of people over 60 years of age will be living in developing countries (WHO 2002). Although these pathologies have been known for quite some time and some symptomatic therapies exist, it is still not possible to cure or prevent these disorders. Thus, unless novel therapeutic strategies are developed, the economical and social impact will be enormous. The majority of AD and PD cases is sporadic and is thought to arise from the combination of environmental factors and susceptibility genes in ways that are not fully understood. Thus, these disorders are considered highly complex in similarity to cancer, diabetes, cardiovascular and neuropsychiatric disorders. The complexity of these disorders makes the discovery of therapeutics difficult. Thus, it is essential that we understand how the environment can affect the organisms and result in the development of the disease.

The epigenome comprises the heritable but potentially reversible changes in gene expression that occur in the absence of changes to the DNA sequence itself. These changes are brought about by modifications of chromatin, such as acetylation, methylation, phosphorylation, or ubiquitylation of histones, DNA methylation, and microRNA (miRNA) (Dolinoy and Jirtle 2008; Fig. 22.1).

Environmental exposure to nutritional, chemical, physical, as well as intellectual or social factors can alter gene expression, and affect adult phenotype by changes in epigenetic modifications at labile genomic regions (Kovalchuk 2008).

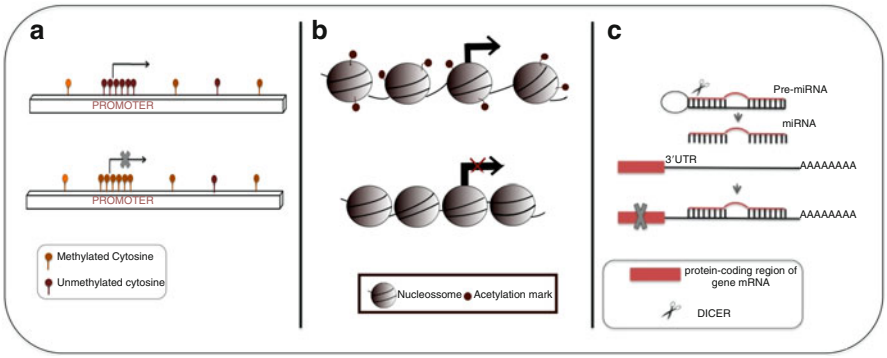

Fig. 22.1 Epigenetic regulation of gene expression. (**a**) DNA methylation. The addition of methyl groups in CpG dinucleotides, that when clustered in CpG islands around the promoters, represses transcription. (**b**) Histone acetylation. One type of histone modifications, which promotes the opening of chromatin around the genes, enables the access of transcriptional machinery. (**c**) miRNA is cut out of a precursor *hairpin-shaped* pre-miRNA to form a mature miRNA, which binds to the 3′ untranslated region (3′ UTR) of a target gene messenger RNA and turns off its activity (***a*** *and* ***b*** *adapted from Marques et al. 2010;* ***c*** *based on an illustration from Victor Ramblos*)

Although it is plausible that epigenetic regulation plays a role in gene expression in AD and PD, the focus on epigenetic effects in these disorders is only now beginning to grow and, to date, very few definitive clues have been discovered.

22.2 Parkinson's Disease

22.2.1 Epidemiology

PD is the second most common neurodegenerative disorder after AD and has an average age of onset of 60 years. According to the Parkinson's Disease Foundation, it currently affects more than four million people worldwide. The prevalence of PD in industrialized countries is generally estimated at about 1–2% of people over 60 years of age. This figure increases to 3–5% in people above 85 years old (Lau and Breteler 2006).

22.2.2 Pathology

PD is characterized by resting tremor, slow and decreased movement (bradykinesia), muscular rigidity, and postural instability. Pathologically, PD patients display loss of dopaminergic neurons in the substantia nigra (SN) pars compacta and frequently have Lewy bodies, which are eosinophilic intracellular inclusions, composed of amyloid-like fibers primarily made up α-synuclein (α-syn) (Weintraub et al. 2008).

The Braak staging hypothesis for PD posits that pathology evolves in 6 stages. In stages 1–2, a pre-motor period, the typical pathological changes, Lewy neurites and Lewy bodies, spread from the olfactory bulb and vagus nerve to lower brainstem regions. The symptomatic period correlates with pathological changes involving the midbrain, including the SN, in stage 3, the mesocortex, in stage 4, and the neocortex in stages 5–6 (Braak et al. 2003).

Other groups have confirmed this pattern of progression of the pathology for the most part. However, some critical evaluation argues that some questions still need to be addressed and additional studies are required (Burke et al. 2008).

22.3 Genetic Linkages

The majority of PD cases are sporadic. However, 5–10% of cases have a defined genetic component, with both recessive and dominant modes of inheritance. Several genes and chromosomal loci, linked to familial forms of parkinsonism and designated as PARK1 to 16, are associated with autosomal dominant, recessive, and

X-linked forms of the disease. There are also loci for which the mode of transmission is still not fully understood due to the limited number of cases identified (Hardy et al. 2009).

The SNCA gene, encoding for α-syn, was the first gene to be associated with familial PD. Three point mutations in the SNCA gene, causing A30P, E46K, or A53T amino acid substitutions in the protein, are known to be linked with autosomal dominant PD. Another mutational mechanism in this gene involves duplication or triplication of the wild-type SNCA gene locus (Klein and Schlossmacher 2007).

Another gene that is responsible for an autosomal dominant form of the disease is the leucine-rich repeat kinase 2 (LRRK2) gene. Some mutations are surprisingly common in certain geographical regions; R1441G for Basque cases, G2019S in Europeans and G2385R and R1628P in eastern Asian people (Farrer 2006). Mutations in both of the dominant genes are associated with the presence of Lewy bodies, the pathognomonic protein inclusions in PD.

Three recessive forms of PD have been identified with mutations in the genes that encode parkin, DJ-1, and PTEN-induced kinase 1 (PINK1). These forms of the disease tend to result in a more selective loss of dopaminergic neurons than that associated with sporadic, late-onset PD. Other genes have been implicated in familial PD, however no pathological identification has been made yet. This is the case for mutations associated with FBXO7, encoding F-box only protein 7 or ATP13A2, encoding probable cation-transporting ATPase 13A2 (Wood-Kaczmar et al. 2006).

22.4 Sporadic Risk Factors

Idiopathic PD, which accounts for about 90–95% of the cases, usually refers to a syndrome characterized by late-onset parkinsonism. The cause of sporadic PD is unknown, but it is considered to result from a combination of environmental and genetic factors. The environmental toxin hypothesis was dominant for much of the twentieth century, and posits that PD-related neurodegeneration results from exposure to a dopaminergic neurotoxin. Living in a rural environment appears to confer an increased risk of PD, perhaps due to increased exposure to pesticide use and wood preservatives. Cigarette smoking and coffee drinking are inversely associated with the risk for development of PD, reinforcing the concept that some environmental factors can modify PD susceptibility (Dauer and Przedborski 2003).

Other factors, such as increased animal fat intake, head trauma, and tobacco smoking have been investigated in relation to PD but no clear correlation has been established yet. Increasing evidence associates the first two factors with an increased risk of PD. In contrast, a negative relationship between tobacco smoking and the development of PD has been observed (Khandhar and Marks 2007).

22.5 The Contribution of Epigenetics

In PD, a direct relation between epigenetics and neurodegeneration has not yet been clearly established, except for miRNA regulation. However, several indirect links between methylation regulators or histone deacetylases inhibitors (HDACi) and the disease mediators have been described.

22.5.1 DNA Methylation

Evidence for DNA methylation role in PD is mainly based on homocysteine (Hcy) cycle dysregulation.

The methyl groups for all biological methylation reactions derive from dietary methyl donors and from cofactors carrying 1-carbon units. In the metabolic cycling of methionine, this compound is converted to the methyl cofactor *S*-adenosylmethionine (SAM). Subsequent to methyl donation, the product *S*-adenosylhomocysteine (SAH) becomes Hcy, which is then either catabolized or remethylated to methionine (Finkelstein 2000; Fig. 22.2).

Variability in levels of Hcy among individuals can result from genetic or environmental factors, with dietary folate levels having a major impact such that there is generally an inverse relationship between plasma folate and Hcy levels (Giles et al. 1995).

Increased concentrations of plasma total Hcy have been reported in patients with PD, which will increase SAH and decrease SAM, leading to an overall decrease in methylation potential (SAM/SAH ratio) (Blandini et al. 2001).

Better cognitive function scores and increased SAM/SAH ratio, has been recently described in patients with PD, thus suggesting a role for methylation in the disorder (Obeid et al. 2009).

A mouse model of PD with a dietary folate deficiency exhibited elevated levels of plasma Hcy and was sensitized to 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) -induced PD-like pathology and motor dysfunction (Duan et al. 2002).

In addition to the Hcy cycle, evidence is now emerging linking DNA methylation levels and genes related to PD, although DNA methylation dysregulation in the disease process was not yet characterized.

A recent study showed that peripheral leukocytes of Japanese PD patients bear fewer short telomeres with constant subtelomeric methylation status in comparison with the healthy controls. Subtelomeric hypomethylation is associated with increased accessibility of DNA-binding proteins for suppression of "telomeric position effect", that is, a mechanism silencing genes neighboring a telomere. The telomeric and subtelomeric regions impaired by oxidative stress progress to become hypomethylated and are open to easy access by oxygen radicals (Maeda et al. 2009).

The PARK2 gene, identified as a mutated target in patients with autosomal recessive juvenile parkinsonism (ARJP), and which has also been accepted as a candidate

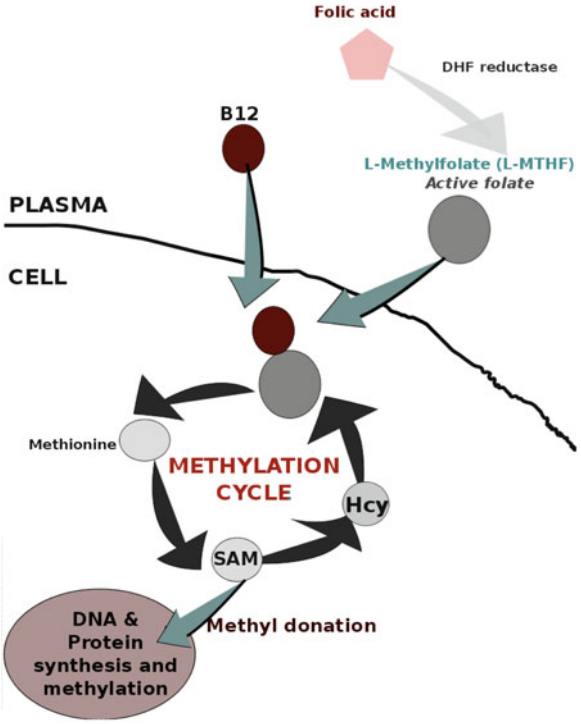

Fig. 22.2 Metabolism of methylation reactions. Folate and vitamin B12 are provided by the diet and are essential for the methylation cycle. Folic acid must be converted into L-methylfolate (L-MTHF) by Dihydrofolate (DHF) reductase in order to be absorbed by the intestinal mucosa. Upon recruitment, L-MTHF associates with Hcy and methionine synthase to produce methionine. This compound is then converted into S-adenosylmethionine (SAM). Through the action of methyltransferases, Hcy is generated and re-enters the cycle or is used to generate methyl groups required for methylation reactions on DNA CpGs or histone lysines/arginines

tumor suppressor gene in several types of cancer, was described to be epigenetically regulated in human leukemia. This could suggest that, similarly to its role on the development of this hematological neoplasm, abnormal methylation and regulation of PARK2 could also play a role in the pathogenesis of PD (Agirre et al. 2006).

Likewise, the SNCA gene is affected by DNA methylation in other pathologies, paving the way to the same dysregulation in PD. An increase in DNA methylation in the promoter of the SNCA gene was described in patients with alcoholism, and was also found to be significantly associated with elevated homocysteine levels and a global DNA hypomethylation and DNA hypermethylation in females with anorexia nervosa (Bönsch et al. 2005; Frieling et al. 2007).

Indeed, the effect of SNCA methylation in PD has recently described. A region of the SNCA CpG island in which the methylation status is altered along with increased SNCA expression was identified in cultured cells. Postmortem brain analysis

also revealed regional non-specific methylation differences in this CpG region in the anterior cingulate and putamen among controls and PD. However, in the substantia nigra of PD, methylation was significantly decreased (Matsumoto et al. 2010). Another group reported a decreased methylation of SNCA intron 1 in the SN, putamen, and cortex of sporadic PD patients, pointing again towards epigenetic regulation of SNCA expression in PD (Jowaed et al. 2010). Subsequently, a reduction of nuclear DNA methytransferase 1 (Dnmt1) levels in human postmortem brain samples from PD and DLB patients, as well as in the brains of α-syn, transgenic mice models was demonstrated. The cytoplasmic sequestration of Dnmt1 resulted in global DNA hypomethylation in human and mouse brains, involving CpG islands upstream of SNCA as well as other genes (Desplats et al. 2011).

Epigenetic regulation of SNCA has also been reported in a familial case of PD, SNCA showed monoallelic expression in lymphoblastoid cell line and in the blood cells of a patient heterozygous for p.Ala53Thr and the epigenetic silencing of the mutated allele involved histone modifications but not DNA methylation. The steady-state mRNA levels deriving from the normal SNCA allele in this patient exceeded those of the two normal SNCA alleles combined, in matching, control individuals (Voutsinas et al. 2010).

22.5.2 Histone Modifications

Several evidences have now proven that chromatin remodeling has a central role in some of the long-lasting effects of dopamine on brain function (Brami-Cherrier et al. 2005). Consequently, the interest in the study of histone modifications and their effect on PD-related toxicity has been increasing.

Starting with one of the known risk factors for PD, pesticide exposure, it was observed that dieldrin induced in mesencephalic dopaminergic neuronal cells a time-dependent increase in the acetylation of core histones H3 and H4 indicating that acetylation is an early event in dieldrin neurotoxicity. The hyperacetylation was attributed to dieldrin-induced proteasomal dysfunction, resulting in the accumulation of a key histone acetyltransferase (HAT), the cAMP response element-binding protein -binding protein (CREBBP or CBP). In mouse models, exposure to dieldrin induced histone hyperacetylation in the striatum and SN. An HAT inhibitor, anacardic acid, significantly attenuated dieldrin-induced histone acetylation as well as the apoptotic cascade that includes caspase-3 activation, Protein Kinase Cδ proteolytic activation and DNA-fragmentation, having a neuroprotective effect against dieldrin-induced nigral dopaminergic neuronal degeneration in primary mesencephalic culture models, which was independent of its antioxidant effect (Song et al. 2010).

Parkin, encoded by PARK2, is a ubiquitin E3 ligase which accumulates at the centrosome in a microtubule-dependent manner in response to proteasome inhibition. The centrosome recruitment of parkin is mediated by its direct binding to histone deacetylase 6 (HDAC6), through multiple interaction domains (Jiang et al. 2008).

The most widely used treatment in PD, Levodopa, (L-DOPA) is associated with motor complications, such as motor fluctuations and dyskinesias (Fabbrini et al. 2007). Dopamine depletion and subsequent L-DOPA treatment has also been associated with profound alterations in posttranslational modifications of striatal histones. In animal models of L-DOPA-induced dyskinesia (LID), dopamine depletion was associated with a reduction in histone H3 trimethylation at Lys 4, while chronic L-DOPA therapy leading to hyperkinesia was marked deacetylation of striatal histone H4 at Lys 5/8/12/16. This study highlighted the presence of histone modifications and supported the hypothesis that chromatin remodeling could contribute to the development and maintenance of LID in PD (Nicholas et al. 2008).

Another study regarding L-DOPA-induced dyskinesias, focused on the dopamine D1 receptor, which has been shown to be critical for the development of this side effect in mice. H3 phosphoacetylation was blocked by D1 receptor inactivation, suggesting that inhibitors of H3 acetylation and/or phosphorylation could be useful in preventing or reversing dyskinesia (Darmopil et al. 2009).

Several studies have focused on the protective effect of HDACi in PD pathology.

An important study in laboratory models showed that the neurotoxicity of α-syn in the nucleus could be rescued by the administration of HDACi in both cell culture and transgenic flies. α-syn could bind directly to histones, reducing the level of acetylated histone H3 in cultured cells and inhibiting acetylation in HAT assays (Kontopoulos et al. 2006). Valproic acid (VPA) administered daily in the diet of an animal model of PD, in a dose that was described to lead to a significant inhibition of HDAC activity and to an increase of histone H3 acetylation in brain tissues, resulted in the prevention of the decrease of the dopaminergic marker tyrosine hydroxylase (TH) in SN and striatum. It also significantly counteracted the death of nigral neurons and the 50% drop of striatal dopamine levels caused by rotenone administration. VPA treatment could also counteract alterations observed in α-syn, being the native form decreased in SN and striatum of rotenone-treated rats, while monoubiquitinated one increased in the same regions (Monti et al. 2010). Chen et al. (2006) showed that VPA pretreatment could upregulate the expression of neurotrophic factors and protect midbrain DA neurons from LPS or 1-methyl-4-phenylpyridinium (MPP+)-induced neurotoxicity. The same authors, posteriorly observed that sodium butyrate (SB) and trichostatin A (TSA), mimicked the survival-promoting and protective effects of VPA on DA neurons in neuron-glia cultures. Similarly to VPA, they increased Glial-derived and brain-derived neurotrophic factors (GDNF and BDNF, respectively) transcripts in astrocytes in a time-dependent manner with a simultaneous increment in promoter-associated histone H3 acetylation, being the time-course for acetylation similar to that for gene transcription (Wu et al. 2008).

TSA was also tested in several dopaminergic neuronal cell lines and a single TSA treatment resulted in decreased cell survival and increased apoptosis in dopaminergic neuronal cells. Pre-treatment with TSA resulted in exacerbated neurotoxic damage to dopaminergic neurons induced by MPP+ and rotenone suggesting that HDACi could influence PD pathogenesis by inhibiting survival and increasing vulnerability of dopaminergic neurons to neurotoxins (Wang et al. 2009).

Interestingly, pharmacological inhibition of Sirtuin 2 (SIRT2), a member of the HDAC family, rescues α-syn-induced toxicity and modifies inclusion morphology in cellular and animal models of PD (Outeiro et al. 2007).

22.5.3 miRNAs

Several microRNAs (miRNAs) and their correspondent targets have been identified in PD, providing new insights about the possibility of using RNA interference as a therapeutical approach for the disease.

A study based on familial forms of PD, studying around 800 families, showed a strong association between rs12720208 in the 3′untranslated region (UTR) of FGF20 and the risk of developing PD. This single nucleotide polymorphism (SNP) disrupted a binding site for microRNA-433 (miR-433), increasing translation of FGF20 in vitro and in vivo. In a cell-based system and in PD brains, this increase in translation of FGF20 was correlated with increased α-syn expression. Overexpression of miR-433b in primary midbrain cultures also prevented dopaminergic differentiation whereas its inhibition resulted in increased TH positive cells (Wang et al. 2008). miRNA expression of midbrain from PD patients was determined, also revealing changes in the expression of miR-133b (de Mena et al. 2010).

In the α-syn(A30P) transgenic mice, a decline in miR-132 levels was observed (Gillardon et al. 2008). This miRNA was previously shown to increase following growth factor administration or neuronal depolarization in vitro (Vo et al. 2005). This decrease in PD could thus represent a molecular signature for a concomitant decrease in neurotrophic and/or neuronal activity in the affected brainstem.

microRNA-7 (miR-7), which is expressed mainly in neurons, represses α -synuclein protein levels through the 3′ UTR of α-syn mRNA. miR-7-induced down-regulation of α-syn could protect cells against oxidative stress. Further, in the MPTP-induced model of PD, miR-7 expression decreased, possibly contributing to increased α-syn expression (Junn et al. 2009). miR-7 inhibits cellular susceptibility of neuroblastoma cells to oxidative stress induced by a mutant form of SNCA, providing evidence that miRNAs protect neuronal cells against cellular stress. The presence of miR-7 in the SN is also verified thus supporting a physiological role in dopaminergic neurons (Bak et al. 2008).

As stated above, miRNAs could be explored as therapeutic targets for PD, however for specific targeting of miRNAs, it will be necessary to address the physiological relevance of these molecules.

22.6 Alzheimer's Disease

22.6.1 Epidemiology

Currently, it is estimated that one person in eight (13%) above 65 years old is diagnosed with AD. The prevalence increases to approximately 40% after the age of 85. The number of AD patients is estimated to reach 7.7 million in 2030, constituting a greater than 50% increase from the 5.1 million aged 65 and older who are currently affected. By 2050, this number is projected to reach between 11 million and 16 million if the current state of medical therapeutics is maintained (Alzheimer's Association 2009).

22.6.2 Pathology

The major clinical hallmarks of AD are progressive impairment in memory, judgment, decision-making, orientation, and language. Diagnosis is based on neurologic examination and imaging, along with the exclusion of other causes of dementia. However a definitive diagnosis can be made only at autopsy to detect the histopathological features of the disease, which include senile plaques, composed by insoluble amyloid-β (Aβ) peptide that accumulates extracellularly, intracellular neurofibrillary tangles composed of hyperphosphorylated tau protein and selective neuronal loss. These plaques and tangles are found predominantly in the frontal and temporal lobes, including the hippocampus. In more advanced cases, the pathology extends to other regions of cortex, including the parietal and occipital lobes (Minati et al. 2009).

22.7 Genetic Forms of AD

Early onset familial AD accounts for less than 1% of all AD cases (Lambert and Amouyel 2007). The genes implicated in these forms of the disease are the genes encoding for amyloid precursor protein (APP), located on chromosome 21q21, the gene encoding for presenilin 1 (PSEN1), located on chromosome 14q24.3, and that encoding for presenilin 2 (PSEN2), on chromosome 1q31–q42 (Ertekin-Taner 2007).

APP mutations account for <0.1% of AD patients (13). Mutations in APP are located near the cleavage sites of the protein resulting in increased production of the Aβ peptide. The average age of onset for this mutation is between mid 40s and 50s but can be modified by the apolipoprotein E (APOE) genotype.

Missense mutations within the PSEN1 gene account for 18–50% of the early-onset autosomal dominant forms of AD and lead to a particularly aggressive form of the disease with an age of onset between 30 and 50 years, which is not influenced by the APOE genotype. The majority of PSEN mutations are single-nucleotide substitutions, but small deletions and insertions have been described as well. All mutations within PSEN1 increase production of the Aβ42, the most toxic form of the peptide. Mutations within PSEN2 have a variable age of onset (40–80 years), appear not to be influenced by APOE and result in increased Aβ production as well (Ray et al. 1998; Bettens et al. 2010).

22.8 Sporadic Risk Factors

The majority of AD cases have complex etiology due to both environmental and genetic factors, which alone do not seem sufficient for causing disease.

The APOE gene, on chromosome 19q13, and its variants, is recognized as a major risk factor in AD, modify the risk of Late Onset AD (LOAD).

The APOE gene contains three common polymorphisms – ε2 (cysteines at codon 112 and codon 158), ε3 (cysteines at codon 112), and ε4 (arginine at codon 112). APOE ε3 is the most frequent form (78%), APOE ε4 makes up 15% and APOE ε2 approximately 7% (Tanzi and Bertram 2001). Analysis of these polymorphisms in normal control populations and in patients with AD has shown that ε4 allele frequency in AD is approximately 40%, compared to 15% in normal and the frequency of the ε2 allele decreased from 10% to about 2% in AD. A dose-dependent relationship between the number of copies of ε4, and the age-of-onset of AD such that ε4/ε4 subjects have an earlier onset than do heterozygous ε4 subjects. Subjects with an ε2 allele, on the other hand, have a later onset (St George-Hyslop and Petit 2004).

Nevertheless, only less than 50% of non-familial AD cases are carriers of the APOE ε4 allele. Therefore, APOE ε4 is not a deterministic factor for the development of the disease and other genes must confer susceptibility to AD.

Some candidate susceptibility genes are the ones encoding α2-macroglobulin (α2M), low-density lipoprotein receptor-related protein (LRP), angiotensin converting enzyme (ACE), insulin degrading enzyme (IDE) and others (Rocchi et al. 2003).

In addition to these genetic polymorphisms that increase the risk for developing AD, multiple environmental factors may influence the onset and progression of the disease. The main risk-increasing factors examined in epidemiological studies are associated with life history. In addition to age, which is by far the major risk factor for AD, others have now been extensively correlated, such as hypertension (as a long-term stress of the blood vessel endothelium and walls), diabetes (by vascular changes or insulin deregulation itself), inflammation, obesity, or head injury (Stozicka et al. 2007).

22.9 Epigenetics in AD

In AD, several works are now arising demonstrating direct epigenetic modulation of the disease. However, except for miRNA dysregulation, which has now been extensively described, a lot of research still needs to be done to understand which modifications and in which genes, epigenetic modulation occurs.

22.9.1 DNA Methylation

Some cytosines in the promoter region of the APP gene are frequently methylated in cases ≤70 years old and significantly demethylated in cases >70 years old. These age-related modifications on DNA methylation alter APP expression and consequently can affect the progressive Aβ deposition with aging in the brain (Tohgi et al. 1999).

Several studies are now demonstrating a correlation between dietary factors, the epigenome and AD pathology. The hypothesis was that nutritional deficits could lead to hyperhomocysteinemia (HCY/SAM cycle alteration, which is involved in the transfer of methyl groups) with the consequent decrease of SAM levels. Methyl donor decrease, in turn, could induce demethylation of DNA and this will result in activation and overexpression of genes involved in AD pathology. Indeed, folate, vitamin B12, and SAM are frequently reduced in the elderly. One of the first studies regarding this issue showed that the reduction of folate and vitamin B12 in culture medium could cause a reduction of SAM levels, and an increase in Aβ production (Fuso et al. 2005). The same authors also demonstrated that Vitamin B deprivation could induce hyperhomocysteinemia and an imbalance of SAM and SAH, in association with PSEN1 and BACE up-regulation and Aβ deposition (Fuso et al. 2008).

Vitamin B deficiency also induced hypomethylation of specific CpG moieties in the 5′-flanking region of PSEN1 gene, and this was reverted with SAM supplementation (Fuso et al. 2011a). In line with the changes observed for PSEN1 methylation patterns, DNA methylases (DNMT1, 3a and 3b) and a putative demethylase (MBD2) were differently modulated in the same experimental conditions (Fuso et al. 2011b).

This relation was further explored by developing a triple transgenic/mutant mouse model (APP*/PS1*/CBS*) showing both amyloid deposition and high serum levels of Hcy resultant of deficient CBS (Cystathionine-Beta-Synthase) activity. The study showed that female APP*/PS1*/CBS* mice exhibited significant elevations of Aβ40 and Aβ42 levels in the brain compared with APP*/PS1* double transgenic mice showing that hyperhomocysteinemia can be a risk factor for AD (Pacheco-Quinto et al. 2006).

Another connection with this nutritional regulation of epigenetic alterations described a downregulation of the neuronal PP2A methyltransferase (PPMT), along with a decrease in PP2A methylation in affected brain regions from AD patient (Sontag et al. 2004). Taking this into account, it was recently observed that hyperhomocysteinemia induced in mice by feeding a high-methionine, low folate diet was associated with increased brain SAH levels, with a reduced PP2A methylation levels, and with tau and APP phosphorylation. These results supported the hypothesis that impaired Hcy metabolism and deregulation of critical methylation reactions can trigger the accumulation of phosphorylated tau and APP in the brain, a process that may favor neurofibrillary tangle formation and amyloidogenesis (Sontag et al. 2007).

Dietary and environmental factors, which are present from as early as the prenatal phase, have a profound impact on our epigenome and may affect diseases developed later in life. Expression of AD-related genes (APP, BACE1) was found to be elevated in aged (23-year-old) monkeys exposed to lead (Pb) as infants, along with a decrease in DNA methyltransferase activity and higher levels of oxidative damage to DNA. These data suggest that AD pathogenesis is influenced by early life exposures and argue for both an environmental trigger and a developmental origin of AD, being the intermediate DNA methylation a form of epigenetic imprinting (Wu et al. 2008).

The age-related epigenetic modifications in AD were further explored in two studies. The first one presented a straight evidence of epigenetic involvement in AD pathogenesis by showing an age-specific epigenetic drift in late-onset AD. PSEN1 and APOE, which participate in Aβ processing, methylenetetrahydrofolate reductase (MTHFR) and DNMT1, which are responsible for methylation homeostasis, presented a significant inter-individual epigenetic variability in the brain and lymphocytes of these patients, which could contribute to late-onset AD predisposition (Wang et al. 2008). Longevity related genes were investigated with respect to promoter methylation in peripheral blood in relation to gender, age and AD. Only one of the genes, HTERT, was shown to be hypermethylated in AD compared to aged normal people and is, by opposite to the normal effect of methylation on gene expression, activated by this epigenetic modification. Consequently, these results indicated a higher telomerase activity probably due to telomere and immune dysfunctions involved in AD pathogenesis (Silva et al. 2008).

22.9.2 Histone Modifications

In contrast to DNA methylation studies, very few studies were aimed at characterizing chromatin modifications in AD. A recent study showed that acute treatment of a mouse model of AD with a HDACi had an effect on memory impairment (Francis et al. 2009).

Chronic HDACi (VPA, SB and vorinostat) injections (2–3 weeks) in APPswe/PS1dE9 mice completely restored contextual memory. The newly consolidated memories were stably maintained over a 2-week period. All HDACi affected class I HDACs (HDAC1, 2, 3, 8) with little effect on the class IIa HDAC family members (HDAC4, 5, 7, 9) (Kilgore et al. 2010).

In another study, systemic administration of the 4-phenylbutyrate (PBA) reversed spatial learning and memory deficits in the Tg2576 mouse without altering Aβ burden. However, the phosphorylated form of tau was decreased in the mouse brain after 4-PBA treatment, along with an increase in the inactive form of the glycogen synthase kinase 3beta (GSK3beta) (Ricobaraza et al. 2009). The same authors, then showed that 4-PBA administration reinstated fear learning in the same animal model of AD, independently of the disease stage: both in 6-month-old Tg2576 mice, at the onset of the first symptoms, but also in aged, 12–16-month-old mice, when amyloid plaque deposition and major synaptic loss has occurred. Reversal of learning deficits was associated with a clearance of intraneuronal Aβ accumulation, and alleviation of endoplasmic reticulum (ER) stress. The expression of plasticity-related proteins were also significantly increased by PBA (Ricobaraza et al. 2010).

Another type of chromatin modifiers, HAT such as CBP, have also been implicated in AD pathology. In primary neurons, CBP is specifically targeted by caspases and calpains at the onset of neuronal apoptosis, and CBP was further identified as a new caspase-6 substrate. This ultimately impinged on the CBP/p300 HAT activity that

decreased with time during apoptosis entry, whereas total cellular HAT activity remained unchanged. Consequently, histone acetylation levels decreased at the onset of apoptosis. Interestingly, CBP loss and histone deacetylation were observed in two different pathological contexts: APP-dependent signaling and amyotrophic lateral sclerosis model mice, indicating that these modifications are likely to contribute to neurodegenerative diseases (Rouaux et al. 2003).

Aβ accumulation, which plays a primary role in the cognitive deficits of AD, interferes with CREB activity. Restoring CREB function via brain viral delivery of the CBP improved learning and memory deficits in the triple transgenic model of AD. These occur without changes in Aβ and tau pathology, and were linked to an increased level of BDNF (Caccamo et al. 2010).

Despite this link between AD and histone deacetylation, there is no evidence that the expression of AD-related genes is affected by chromatin modulation in AD. To close this gap, it will be important to undertake a more global study looking at the epigenetic control of the expression of AD-related genes by histone modifications in AD samples and in cell and animal models. In this way, we would develop a better understanding of the role of chromatin remodeling in AD pathogenesis.

22.9.3 miRNAs

miRNAs are, by far, the most studied epigenetic modification in AD. The expression of several blood mononuclear cells (BMC) miRNAs was found to increase in AD relative to normal elderly controls levels, and could differ between AD subjects bearing one or two APOE4 alleles. miRNAs significantly upregulated in AD subjects were miR-34a and 181b (Schipper et al. 2007).

miRNAs belonging to the miR-20a family (that is, miR-20a, miR-17-5p and miR-106b) can regulate APP expression in vitro and at the endogenous level in neuronal cell lines. A tight correlation between these miRNAs and APP was found during brain development and in differentiating neurons. This was further corroborated by the observation that a statistically significant decrease in miR-106b expression was found in sporadic AD patients (Hébert and De Strooper 2009).

miR-107 levels decreased significantly even in AD patients with the earliest stages of pathology. Computational analysis predicted that the 3′-untranslated region (UTR) of BACE1 mRNA is targeted multiply by miR-107. BACE1 mRNA levels tended to increase as miR-107 levels decreased in the progression of AD. Cell culture reporter assays performed with a subset of the predicted miR-107 binding sites indicate the presence of at least one physiological miR-107 miRNA recognition sequence in the 3′-UTR of BACE1 mRNA Wang et al. (2008).

miR-29a, -29b-1, and –9 can regulate BACE1 expression in vitro. The miR-29a/b-1 cluster was significantly decreased in AD patients displaying abnormally high BACE1 protein. Similar correlations were found during brain development and

in primary neuronal cultures. The same authors provided evidence for a causal relationship between miR-29a/b-1 expression and Aβ generation in a cell culture model (Hébert and De Strooper 2009).

Another study predicted that miR-298 and miR-328 recognize a specific binding site in the 3′-UTR of BACE1 mRNA and exert regulatory effects on BACE1 protein expression in cultured neuronal cells (Boissonneault et al. 2009).

In APPswe/PS_E9 mice and age-matched controls, the expression of miR-34a is inversely correlated with the protein level of bcl2 and its expression directly inhibited Bcl2 translation in SH-SY5Y cells. Higher levels of active caspase-3 were observed in a stable transfectant cell line of miR-34a or in the APPswe/Ps mice compared to controls. Consistently, miR- 34a knockdown increased the level of bcl2 protein in SH-SY5Y cells, which was accompanied by a decrease of active caspase-3. These findings suggested the abnormal expression of miR-34a might contribute to the pathogenesis of AD, at least in part by affecting the expression of bcl2 (Wang et al. 2009).

AD brains presented a specific up-regulation of an NF-kB-sensitive miRNA-146a highly complementary to the 3′UTR of complement factor H (CFH), an important repressor of the inflammatory response of the brain. Up-regulation of miRNA-146a coupled to down-regulation of CFH was observed in AD brain and in interleukin-1β, Aβ42, and/or oxidatively stressed human neural (HN) cells in primary culture (Lukiw et al. 2008).

22.10 Conclusions

In complex disorders, such as AD and PD, which have a multifactorial origin, the understanding of the causes or risk factors underlying the sporadic cases of these disorders remains difficult. Epigenetics may represent the missing link in the interplay between genes and environment, providing possible clues to understand the etiology of these complex diseases. Despite the increasing number of evidences about the involvement of epigenetic mechanisms in the pathology of AD and PD, these are still early days in understanding how epigenetic modulation occurs during the neurodegenerative process and the impact of these changes in the pathological hallmarks and general deregulation occurring in AD and PD. If these changes in the epigenome continue to be unraveled, it may be possible, in the near future, to intervene therapeutically by modulating the epigenetic changes associated with these disorders.

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Chapter 23
Cellular Redox, Epigenetics and Diseases

Shyamal K. Goswami

Abstract In the past decade, epigenetic regulation has emerged as the nodal point of modulating gene expression in eukaryotes. It involves structural reorganization of the chromosome, primarily through post translational modifications of the histones and methylation of cytosine residues in the DNA. Other aspects of DNA functions such as DNA replication and repair also come under the larger ambit of epigenetic regulation. Reactive oxygen/nitrogen species ($R^O/_NS$) has long been perceived as deleterious agents affecting cellular functions, causing degenerative diseases. However, during the past decade akin to kinase-phosphatase signaling, oxidative-reductive modifications of proteins by reactive species, especially at lower concentrations, have also emerged as mediators of key regulatory signals. DNA methyltransferases (DNMTs) catalyze methylation of cytosine residues in the DNA while methionine adenosyltransferase (MAT) synthesize the methyl donor, S-adenosyl methionine (SAM). Both the enzymes have redox sensitive cysteine residues in their catalytic sites. Increased generation of ROS, as it occurs in cancer cells; affects activities of these enzymes, altering the status of DNA methylation and the epigenome. Histone methyl transferases, the key determinant of "epigenetic landscape" also require S-adenosyl methionine (SAM) for their activities. Hence, altered pool of SAM in a more oxidized cellular environment also affects the status of histone methylation and the epigenome. Numerous such evidences have accumulated over the past decade suggesting a close interrelation between cellular redox and gene expression wherein various transcription factors, coactivators and chromatin constituents work in tandem mediating the cognate responses. However, a comprehensive understanding of how these events is coordinately regulated by various RO/NS axis is still emerging and the following chapter is an update in this regard.

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Since the Central dogma of Molecular Biology was proposed about 40 years ago; our understanding of the intricacies of gene regulation has undergone tectonic shifts almost every decade. It is now widely accepted that the complexity of an organism is not directed by the sheer number of genes it carries but how they are decoded by a myriad of regulatory modules. Over the years, it has emerged that the organizations chromatins and its remodeling; splicing and polyadenylation of pre-mRNAs, stability and localization of mRNAs and modulation of their expression by non-coding and miRNAs play pivotal roles in metazoan gene expression. Nevertheless, in spite of tremendous progress in our understanding of all these mechanisms of gene regulation, the way these events are coordinated leading towards a highly defined proteome of a given cell type remains enigmatic. In that context, the structures of many metazoan genes cannot fully explain their pattern of expression in different tissues, especially during embryonic development and progression of various diseases. Further, numerous studies done during the past quarter of a century suggested that the heritable states of transcriptional activation or repression of a gene can be influenced by the covalent modifications of constituent bases and associated histones; its chromosomal context and long-range interactions between various chromosomal elements (Holliday 1987; Turner 1998; Lyon 1993). However, molecular dissection of these phenomena is largely unknown and is an exciting topic of research under the sub-discipline epigenetics (Gasser et al. 1998).

The term epigenetics was first coined in 1938 by the developmental geneticist Conrad Waddington to explain certain aspects of inheritance in cell differentiation and organogenesis (Waddington 1938). Since then, the operational definition of epigenetics remained a subject of intense debate (Haig 2004). As an example, while molecular biologists try to explain epigenetic regulation of gene function through the modifications of DNA and histones (Riggs et al. 1996), others consider that factors as diverse as hormones and temperature are epigenetic regulators (Herring 1993). According to the most recent definition of epigenetics, as proposed by Berger et al., "an epigenetic trait is a stably heritable phenotype resulting from changes in a chromosome without alterations in the DNA sequence" (Berger et al. 2009). Also, as per their nomenclature, establishment of a stably heritable epigenetic state involves three classes of signals viz., "*Epigenator*, which emanates from the environment and triggers an intracellular pathway; an *Epigenetic Initiator*, which responds to the *Epigenator* and is necessary to define the precise location of the epigenetic chromatin environment; and an *Epigenetic Maintainer* signal, which sustains the chromatin environment in the first and subsequent generations" (Berger et al. 2009).

In that context, various studies over the past decade have shown that reactive oxygen/nitrogen ($R^O/_NS$) species can also influence epigenetic state of a genome. However, since the biology of reactive oxygen/nitrogen species ($R^O/_NS$) is highly complex and diverse (Heo 2011), their influence upon epigenetic events are poorly defined as yet. Therefore, perhaps it is too early to categorize their role in epigenetics in the proposed framework of "*Epigenator-Epigenetic Initiator and Epigenetic Maintainer"*. With that background, in the following sections I will summarize some of the recent developments in understanding the role of $R^O/_NS$ in epigenetic regulation. Epigenetic regulation involves structural reorganization of the chromosome that

is primarily mediated by the methylation of cytosine residues in the DNA and post translational modifications of the histones. Also, although epigenetic regulation generally implies altered transcriptional activity, other aspects of DNA functions that is DNA replication and repair also come under the purview of epigenetic regulation.

23.1 Intracellular $R^O/_NS$ and Redox Signaling

Intracellular generation of free radicals are attributed to a plethora of pro-oxidant enzymes such as NADPH and xanthine oxidases, nitric oxide synthases, cyclooxygenases, mitochondrial electron transport complexes, and Fenton reaction. In the dynamic cellular milieu, while the pro-oxidant enzymes continuously generate free radicals and $R^O/_NS$, various antioxidant systems attenuate them, maintaining redox homeostasis (Heo 2011; Antelmann and Helmann 2011). Such controlled, limited generation of free radicals and $R^O/_NS$ contribute to normal physiological processes such as cell signaling, cell cycle progression and embryonic development (Heo 2011; Antelmann and Helmann 2011; Boivin et al. 2010). However, under certain pathophysiological conditions, such balance is tilted towards aberrant pro-oxidant activities, resulting in more oxidized intracellular environment, a condition termed as oxidative stress. Oxidative stress has been implicated in a plethora of diseases like cancer, diabetes, inflammatory, neurodegenerative and cardiovascular disorders (Nediani et al. 2011; Pamplona et al. 2008; Weaver 2009). However, despite the existence of ample evidences supporting the oxidative stress theory of diseases, there are many discrepancies in the interpretation of results compiled over the past two decades (Janssen-Heininger et al. 2008). In accordance, antioxidant therapies for curing those diseases have also shown mixed results, further arguing against generalization of oxidative mechanisms of disease progression (Gutteridge and Halliwell 2010). It now appears that rather than causing oxidative damage only, $R^O/_NS$ can also cause transient modifications of specific targets regulating their functions (Weaver 2009; Dansen et al. 2009). However, due to their extremely short half-life and the transient nature of such oxidative/ nitrosative modifications they impart upon their targets, biochemical mechanisms of redox regulation of gene expression are just emerging (Woo et al. 2010). Also, with the availability of newer techniques for the detection and characterization of various $R^O/_NS$, certain earlier observations regarding redox regulation of gene expression have been revisited (Banerjee et al. 2010; Gloire and Piette 2009); while several novel concepts have emerged (Upham and Trosko 2009).

Reversible phosphorylation of serine, threonine and tyrosine residues in signaling proteins play a nodal role in cellular processes like cell proliferation, differentiation, adhesion, survival etc. A plethora of growth factor, cytokines and hormones use such phosphorylation-dephosphorylation mechanisms for transmitting respective signals from the cell surface to the nucleus (Gough and Foley 2010). During the past decade akin to kinase-phosphatase signaling, oxidative-reductive modifications of regulatory proteins have also emerged as mediators of certain signals (Weaver

2009; Dansen et al. 2009; Woo et al. 2010; Upham and Trosko 2009). However, due to inadequate knowledge about the targets and the types of modifications, mechanisms of redox signaling are poorly understood and number of laboratories are developing methodologies for analyzing redoxproteomics under various pathophysiological contexts (Bregere et al. 2008; Dabkowski et al. 2010; Burgoyne and Eaton 2010). Emerging evidences also suggest that amongst various oxidative modifications of regulatory proteins, S-glutathionylation, -sulfenation and -nitrosylation are most prevailing and important in mediating redox-signals under various pathobiological contexts (Dulce et al. 2011; Chen et al. 2010; Maller et al. 2011; Nishida et al. 2011; Kornberg et al. 2010).

23.1.1 Cellular Redox and DNA Methylation

In eukaryotes, post-transcriptional modifications of RNA occur quite extensively, especially in tRNAs. On the contrary, only modification that occurs in DNA bases is the methylation of cytosine residues in the CpG dinucleotides. Overall occurrence of CpG sequences in mammalian genomes is much less (~20%) than that is expected for their random occurrence in the genome. They often occur as clusters (CpG islands) in the regulatory regions of many protein coding genes, especially those which are constitutively expressed with housekeeping functions (Xie et al. 2011). Cells have dedicated enzymatic machinery ensuring methylation of cytosine residues in CpG sequences. Normally, many of the C residues in the CpG islands remain unmethylated or hypomethylated; while under certain conditions their methylation leads to chromatin condensation and transcriptional repression (Easwaran et al. 2010). Methylation of cytosine residues in the CpG islands inhibits the binding of certain transcription factors like CREB but enhances the binding of certain others like C/EBPα and methyl-CpG binding domain proteins like MBP (Pierard et al. 2010; Joulie et al. 2010; Rishi et al. 2010). Upon binding, MBPs act as recruitment points for histone modifying enzymes. Thus, methylation of CpG residues plays a major role in chromosome structure and function. Recent studies suggest that CpG methylation also plays a role in regulating the expression of micro RNAs (Lujambio and Esteller 2009).

DNA methyltransferases (DNMTs) catalyze methylation of cytosine by transferring the methyl groups of S-adenosylmethionine (SAM) to the 5th position of the pyrimidine ring. In mammals, there are three DNA methyltransferases viz., DNMT1, DNMT3a and DMNT3b (Cheng and Blumenthal 2008). Following replication, DNMT1 methylates the newly synthesized strand of DNA, thus carrying out "Maintenance methylation". It is expressed at a high level in the S phase of cell cycle and has high affinity for CpG dinucleotides where only one strand is methylated (hemimethylated DNA). The other two DNA methyl transferases i.e., DNMT3a and DNMT3b can carry out *de novo* methylation of hemimethylated and unmethylated DNA and thus play more active roles in gene silencing. Novel regulatory pathways in plants and mammals are now emerging wherein small interfering RNAs modulate transcriptional silencing via DNA methylation (He et al. 2011).

Since the development and propagation of cancer involves extensive epigenetic dysregulation, cancer cells are often used as a paradigm of understanding epigenetic mechanisms (Brower 2011). In cancer cells, cellular metabolism is substantially altered which in turn perturb the redox homeostasis (Cairns et al. 2011; Tew and Townsend 2011). The ratio of reduced versus oxidized glutathione (GSH/GSSG) is very high in normal cells so that the reduced cellular milieu is maintained. Cancer cells have higher levels of free radicals and reactive oxygen/nitrogen species that substantially diminish the level of reduced glutathione, resulting in a more oxidized environment (Tew and Townsend 2011; Finley et al. 2011). Such oxidized cellular environment affects enzymatic reactions, especially those requiring redox-sensitive cysteine residues (Guttmann 2010). Methionine adenosyltransferase (MAT), the key enzyme involved in the generation of SAM, is inhibited by free radicals (Avila et al. 1998; Lindermayr et al. 2006). Since SAM is the methyl donor to the cytosine residues, decrease in SAM affects DNA (and histone) methylation. DNA methyltransferases also have redox sensitive cysteine residues in their active sites and in oxidized cellular environment, function of DNMT are also directly affected due to the oxidation of those cysteines (Svedružić and Reich 2005).

Taken together, substantial evidences suggest that $R^O/_NS$ might act as a epigenetic regulator via DNA methylation. However, except its potential inhibitory effect on MAT and DNMT, very little mechanistic details are known till date. It is likely that $R^O/_NS$ might regulate DNA methylation by other mechanisms as well. As an example, arsenic, a genotoxic agent; induces oxidative stress and causes hypermethylation of DNA; resulting in cancer, diabetes, cardiovascular and neurological disorders, thereby acting more as an epigenetic regulator than a classical mutagen (Flora 2011). However, whether or not the epigenetic effect of arsenic is a consequence of oxidative injury is not known yet (Coppin et al. 2008). Another lesser known connection between $R^O/_NS$ and DNA methylation is, excessive generation of $R^O/_NS$ leads to DNA damage; extent of which is measured by the formation of 8-oxo-7, 8-dihydroguanine (8-oxoGua). In cancer cells, a positive correlation has been found between level of 8-oxoGua and the extent of hypomethylation of DNA. However, the mechanistic interrelationship is unknown (Guz et al. 2008).

23.1.2 Cellular Redox and Histone Methylation

Epigenetic regulation requires coordination between the cytosine methylation (in CpG islands) and that of lysine$^{4\ \&\ 9}$ of histone H3. Methylation of these two lysine residues is carried out by histone methyltransferases and their modifications have reciprocal correlations with that of DNA. While methylation of Lys^4 prevents cytosine methylation, that at $Lysine^9$ facilitates it (Cheng and Blumenthal 2010). Histones can also be methylated at other lysines *viz*., those at 27, 36 and 79 of histone H3 and at 20 of histone H4. Also, depending upon the methyltransferase, lysine residues are mono-, di-, or tri-methylated, each conferring specific functional properties to that histone molecule they target (Upadhyay and Cheng 2011). Histone and DNA methylation, in conjunction with other histone modifications create an

"epigenetic landscape" that facilitates genome wide recruitment of various gene regulatory modules dictating cell proliferation, differentiation, stemness as well as development and progression of various degenerative diseases (Rakyan et al. 2011). Although till date a direct relationship between cellular redox and histone methylation has not been reported, existence of such correlations is very likely. All known histone methyl transferases with the exception of Dot1, contain an evolutionarily conserved SET domain comprising of ~130 amino acids. Like DNA methyl transferases, histone methyl transferases also require S-adenosyl methionine (SAM) for their activities and as discussed above, cellular pool of SAM can be affected by increased levels of reactive species (Svedruzić and Reich 2005). Furthermore, at least a subset of histone lysine methyltransferases i.e., SUV39 subfamily, contains multiple conserved cysteine residues required for maintaining the 3D structure of the catalytic sites (Zhang et al. 2003). So, it is likely that at least some if not all histone lysine methyltransferases might be sensitive to cellular redox. Also, histone lysine methylation is a reversible process wherein demethylation lysine$^{4\,\&\,9}$ (mono- and di-methyl) of histone 3 is catalyzed by lysine specific demethylase-1, a flavin-dependent amine oxidase. Hene demethylation of lysines is more likely to be affected by changes in cellular redox milleu (Upadhyay and Cheng 2011). Since certain diseases like cancer involves a significant alterations in cellular redox environment as well as extensive epigenetic modifications, understanding correlations between histone methylation and ROS might further enhance our understanding of cancer biology (Varier and Timmers 2011).

23.1.3 Cellular Redox and Chromatin Organization

Structural organization of chromatin is a highly dynamic process involving a myriad of DNA protein and protein-protein interactions. It also involves a large number of post translational modifications of histones, non-histone chromosomal proteins, transcription factors, coactivators etc.; modulating gene expression, gene silencing and DNA replication (Bell et al. 2011). Considering the pervasive role of cellular redox in mediating various physiological responses, it is likely that $R^{O}/_{N}S$ also affect chromatin dynamics, especially under various pathophysiological conditions. Exposure of mammalian cells to anticancer drugs, DNA adducts, ionizing and ultraviolet (UV) irradiations cause oxidative injury to chromosomes. Early studies in this regard were directed towards understanding whether or not oxidative stress causes chromosomal organization leading towards various diseases (Mitra et al. 2002; Rahman 2002). While looking the mechanisms of neurodegeneration, Bai and Konat demonstrated that H_2O_2 at lower concentration (5 μM) triggers cytoplasmic signaling followed by higher order chromatin degradation that is reversed upon removal of the oxidant (Bai and Konat 2003). In C6 rat glial cells, depletion of glutathione leads to the generation of giant, high molecular weight and internucleosomal DNA fragments prior to cell death (Higuchi and Yoshimoto 2004). When cell death is induced in renal proximal tubular epithelial cells (LLC-PK1) by 2, 3,

5-tris-(glutathione-S-yl) hydroquinone [TGHQ], it generates ROS followed by premature chromatin condensation that requires activation of ERK, phosphorylation of histone H3 and poly (ADP-) ribosylation (Tikoo et al. 2001). Nitric oxide is a potent reactive nitrogen species and a key mediator of various physiological signals. It has been implicated to many diseases and amongst its targets are protein tyrosine residues which it converts into 3-nitrotyrosine. Neutrophils, a major source of nitric oxide, invade murine mutatect tumors and causes selective nitration of tyrosines in histones (Haqqani et al. 2002). Taken together, there are substantial evidences indicating role of $R^O/_NS$ in altering chromatin structure. However, precise biochemical mechanism by which they impart epigenetic regulations is still obscure and sporadic.

23.1.4 Cellular Redox, Histone Acetylation and Chromatin Remodeling

Various small molecules including metabolites, nutrients and therapeutics affect cellular proteome via epigenetic modulation of gene expression (Selvi et al. 2010). Also, at least some of those modulators exert their effects via alteration of cellular redox, although the mechanisms involved are poorly understood as yet (Cyr and Domann 2011). As an example, arsenic has been in use for centuries as a therapeutic agent against cancer and in recent years there has been resurgence in studying its effects on leukemic cells (Chen et al. 2011). At certain concentrations, it has genotoxic effects due to the generation of reactive oxygen species, resulting in various degenerative diseases like cancer. Arsenic induces hypermethylation of DNA (Coppin et al. 2008) and hyper-acetylation of histones H3 and H4 (Perkins et al. 2000). However, its epigenetic effects are yet to be attributed to its ability to induce oxidative stress (Coppin et al. 2008; Perkins et al. 2000). Nevertheless, since it binds to thiol residues and affects redox metabolism; such association is quite plausible (Flora 2011). Many small molecular inhibitors of HDAC activities also mediate their effects by inducing oxidative stress. Exposure of macrophage like cell line MonoMac6 to cigarette smoke extract increases the $R^O/_NS$ level followed by a decrease in HDAC 1–3 activities due to nitrotyrosine and aldehyde-adduct formation (Yang et al. 2006). Such decreased HDAC activities results in the increased expression of cytokine genes like IL-8 and TNF-α. When A549 epithelial cells are treated with either cigarette smoke-conditioned medium, peroxynitrite or hydrogen peroxide; level of $R^O/_NS$ increases followed by nitration of tyrosine^{253} of HDAC2, directing it to proteasomal degradation (Osoata et al. 2009). In neurons, Brain-derived neurotrophic factor triggers NO synthesis which causes S-nitrosylation of histone deacetylase 2 (HDAC2) at Cys^{262} and Cys^{274}. Nitrosylation of HDAC2 does not affect its deacetylase activity but causes its release from chromatin, resulting in increased acetylation of histones which triggers neurotrophin-dependent gene expression (Nott et al. 2008). Nitric oxide-releasing acetylsalicylic acid (NO-ASA) is an anti-inflammatory drug that is cytotoxic to various tumor cell lines. Treatment of human B-lymphoblastoid TK6 cells with NO-ASA leads to the generation of $R^O/_NS$ causing DNA damage and

phosphorylation of histone H2AX at Ser^{139} during S-phase. Prolong exposure to NO-ASA also induces atypical apoptosis characterized by highly condensed chromatin but no nuclear fragmentation (Tanaka et al. 2006). Alpha and beta-unsaturated carbonyl compounds like cyclopentenone prostaglandin and 4-hydroxy-2-nonenal which produce reactive carbonyl species (from the peroxidation of arachidonic acid) carbonylate HDAC1, -2, and -3 at two conserved cysteine residues (Cys^{261} and Cys^{273} in HDAC1), attenuating their activities and resulting in changes in histone H3/H4 acetylation followed by the activation of defensive genes like heme oxygenase-1, Gadd45, and HSP70 (Doyle and Fitzpatrick 2010). There are also instances where reactive species increase HDAC activities. When cardiac myocytes are treated with lipopolysaccharides (LPS), HDAC3 activity is increased via mitochondrial ROS and c-*Src* signaling upregulating TNF-α (Zhu et al. 2010). Treatment L6 myoblasts with insulin under hyperglycemic condition induces methylation (at $Lysine^{4\ \&\ 9}$), phosphorylation (at Ser^{10}) and acetylation of histone H3 by enhanced generation of ROS; highlighting its role in epigenetic regulation of diabetes (Kabra et al. 2009). Taken together, there are ample evidences linking histone acetylation-deacetylation with $R^{O}/_{N}S$, but the mechanistic details are yet to be investigated.

23.1.5 Cellular Redox and Transcription Factor Activities

Since cellular metabolism primarily occurs in the cytosol (and mitochondria), earlier it was believed that while cytosol is more susceptible to oxidative insults, nucleus generally remains in a reduced state. However, recent studies demonstrate that the redox environment in the nucleus is also dynamic (Moldovan and Moldovan 2004). At the early stages of cell proliferation, nuclear content of GSH, a key redox buffer; increases and it co-localizes with the DNA (Diaz Vivancos et al. 2010). A reciprocal relationship between level of nuclear GSH and that of certain transcripts encoding stress and defense proteins has also been reported (Markovic et al. 2010). Although our knowledge about the role of $R^{O}/_{N}S$ in affecting nuclear events like histone modifications and chromatin remodeling is quite limited; substantial progress has already been made in understanding the modulation of transcription factor activities by cellular redox. The first evidence that a transcription factor activity can be regulated by $R^{O}/_{N}S$ came from the observation that oxidation of specific cysteine residues at the DNA binding domain of *c*-JUN and c-FOS, the two components of AP-1 transcription factor; reduces their DNA binding affinities (Abate et al. 1990). Ref-1, a nuclear redox protein can restore the reduced state of c-JUN and c-FOS, along with the DNA binding functions (Xanthoudakis and Curran 1992). Nitric oxide is a potent reactive nitrogen species and a modulator of various physiological responses. It nitrosylates specific cysteine residues of JUN and FOS and induces S-glutathionylation of c-JUN; affecting their DNA binding functions (Nikitovic et al. 1998; Klatt et al. 1999). Subsequent to these early but seminal observations, numerous studies have shown that $R^{O}/_{N}S$ can modulate AP-1 activities by modulating (a) the upstream kinases, (b) expression of its constituent subunits

(i.e., j*un* and *fos*) and (c) its interaction with p300/CBP coactivators (Nelson et al. 2006; Jindal and Goswami 2011; Rahman et al. 2004). It now appears that cellular redox might affect the activities of transcription factors in several ways. With increased generation of $R^{O}/_{N}S$, certain cysteine residues might switch to more oxidized state, resulting in an alteration in its conformation affecting DNA binding and transactivation functions. Also, increased $R^{O}/_{N}S$ might affect their interaction with certain metal ions acting as cofactors; thereby inhibiting their functions (Jindal and Goswami 2011; Tong et al. 2007).

While AP-1 was the first transcription factor shown to be sensitive towards oxidation-reduction *in vitro*; NFκB was the first to be identified eliciting similar responses *in vivo* (Schreck et al. 1991). Since then, NFκB has been a paradigm of understanding role of cellular redox in gene expression (Gloire et al. 2006; Gloire and Piette 2009; Oliveira-Marques et al. 2009). Lung cells are vulnerable to massive oxidative insults as it responds to inhaled pathogens by recruiting ROS-producing macrophages and neutrophils (Fialkow et al. 2007). Inhaled pollutants like cigarette smoke, automobile exhausts etc., also induce oxidative stress in the lung. Excessive generation of $R^{O}/_{N}S$ leads to various inflammatory diseases like chronic obstructive pulmonary disease (COPD), asthma, pulmonary fibrosis and cancer; each with characteristic gene expression programs wherein NF-κB plays a pivotal role (Tasaka et al. 2008). In unstimulated cells, members of the NF-κB family are sequestered in the cytoplasm either as their precursors (p100 and p105) or by the three IκB proteins i.e., IκBα, β and ε. Upon stimulation, IκBs are phosphorylated and degraded by 26S proteasome followed by the translocation of NF-κB subunits to the nucleus (Wertz and Dixit 2010). Phosphorylation of IκBs occurs at specific Serine and Tyrosine residues by various canonical and non-canonical pathways (Gloire and Piette 2009; Oliveira-Marques et al. 2009; Wertz and Dixit 2010). Once in the nucleus, subunits of NF-kB undergo multiple post translational modifications regulating its accessibility and binding to target DNA (Gloire and Piette 2009). Post translational modifications of the p65 subunit of NF-κB have been extensively studied as a paradigm of understanding the biological responses mediated by the NF-κB family. Phosphorylation of p65 occurs at multiple sites of which that at Ser^{276} induces its interaction with CBP/p300 followed by its acetylation at Lys^{310} (Yao et al. 2010; Rajendrasozhan et al. 2008). Increased phophorylation-acetylation of p65 leads to the recruitment of IKKα followed by the phosphorylation of histone H3 at Ser^{10} and its acetylation at Lys^{9} and Lys^{14} by CBP (Yao et al. 2010; Rajendrasozhan et al. 2008). Such phosphorylation-acetylation axis involving p65, IKKα, CBP and HistoneH3 is involved in the activation of pro-inflammatory genes in mouse lung *in vivo* and in human monocyte/macrophage cell line MonoMac6 *in vitro* by pro-oxidant cigarette smoke extract (Rajendrasozhan et al. 2008). Apart from activating numerous genes, NF-κB also represses certain others under specific contexts wherein isoforms of HDAC are recruited by distinct NF-kB complexes (Liu et al. 2010). Pro-oxidant constituents of cigarette smoke extracts decrease the expression level and activity of HDACs, resulting in derepression of those genes (Yang et al. 2006). In agreement with these observations, decrease in HDAC activities due to nitrosylation, nitration and carbonylation (by $R^{O}/_{N}S$) has been observed in smokers

and asthma patients, (Barnes 2009). Taken together, substantial evidences have accumulated over the past 10 years suggesting a close interrelation between cellular redox and gene expression wherein transcription factors, coactivators and chromatin constituents work in tandem mediating the redox response. However, a comprehensive understanding of how these events are coordinately regulated by various reactive oxygen/nitrogen species are still emerging and coming years are likely to shed more light on this novel aspect of epigenetic control (Shlomai 2010).

23.1.6 Cellular Redox and DNA Replication

As in transcription, DNA repair and replication also involves extensive organization of chromatin, wherein both its accessibility and compactness are tightly controlled (Chagin et al. 2010). Wealth of information accumulated over the past quarter of a century has delineated how in response to various external cues, mammalian cells encompass through highly orchestrated cell cycle pathways ensuring fidelity of genome duplication (Corpet and Almouzni 2009; Chakraborty et al. 2011). In this context, although the nodal role(s) of kinases and phosphatases in cell cycle regulation and DNA replication have been well documented (Yu and Cortez 2011); that of $R^{O}/_{N}S$, if any; is largely unexplored. Nevertheless, substantial evidences suggest that upon growth stimuli, low intensity generation of ROS modulate the downstream signals leading towards cell division (Ishimoto et al. 2011). Although an wider role of ROS in cell cycle regulation is yet to be established, evidences accumulated from studies with cancer and other degenerative diseases suggest a close association between generation of ROS and cell division (Matés et al. 2010). Some commentators have even perceived cell cycle as "Redox cycle" (Burhans and Heintz 2009). Apart from limited generation of reactive species modulating physiological signals, excessive generation of $R^{O}/_{N}S$ also leads to DNA damage by altering bases, creating single-strand breaks and abasic sites that are repaired via the base excision repair (BER) pathways that involve complex coordination between transcription, histone modifications and replication (Mitra et al. 2002). Taken together, it is thus likely that ROS might also have a broader role in chromatin organization during genome duplication. However, till date, experimental evidences directly correlating ROS and epigenetic regulation of DNA replication is scanty.

23.1.7 Epigenetic Regulation of Embryonic Development and Cellular Redox

Epigenetic regulation plays a nodal role in embryonic development that involves extensive cell differentiation and organogenesis (Banaszynski et al. 2010). During the development of metazoan organisms, based upon the extent of vascularization; different regions of the embryo gets different levels of oxygen which results in the

creation of distinct repertoires of $R^O/_NS$ like NO, $O_2^{\cdot}$ and H_2O_2. It is now believed that such differential distribution of $R^O/_NS$ plays a key role in epigenetic regulation of tissue development (Dennery 2010). According to a recent study, during heart development, maturation of mitochondria leads to the closure of the permeability transition pores, resulting in decreased levels of reactive oxygen species which in turn influences differentiation of cardiac myocytes (Drenckhahn 2011). During the early stages of development, ROS generated by Nox2 or Nox4, the two NADPH oxidases involved in signal transduction in non-phagocytic cells are essential for chondrocyte differentiation (Kim et al. 2010). Taken together, although such "Free Radical theory of development" is more of a concept (Hitchler and Domann 2007), emerging evidences support a broader roles of $R^O/_NS$ in cell differentiation and tissue development while underlying mechanisms are poorly understood as yet (Ufer et al. 2010).

23.2 Concluding Remarks

Sustenance and propagation of a cell (and an organism) involves a complex choreography of energy generation, metabolic turnover, gene expression, replication and maintenance of the genome, integration of organelle functions etc. Like all other sub disciplines of biology, understanding of cellular redox was initiated almost half a century ago with a compartmentalized approach to understand the energy generation by the mitochondria (Ernster and LEE 1964). However, it evolved with time, and in the process; terms like "Oxidative stress" and "Redox signalling" and Redox homeostasis" emerged with their own connotations. Today, the anticipated role of redox has gone beyond it initial boundary of bioenergetics and has pervaded into territories as diverse as regulation of metabolic enzymes, transcription factors, microRNAs etc. (Shlomai 2010; Simone et al. 2009). In that context, role of epigenetics has also evolved from conceptualization to fine dissection of it mechanisms. As discussed above, the inter connection between cellular redox and epigenetics are still sporadic and coincidental. However, in view of the totality of biological responses wherein both redox homeostasis and epigenetic modulation are key contributors, it is likely that these two are functionally connected. It is thus expected that in coming years more direct and mechanistic details of their interrelationship will be revealed.

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Part V
Understanding of Epigenetics: A Chemical Biology Approach and Epigenetic Therapy

Chapter 24
Stem Cell Plasticity in Development and Cancer: Epigenetic Origin of Cancer Stem Cells

Mansi Shah and Cinzia Allegrucci

Abstract Stem cells are unique cells that can self-renew and differentiate into many cell types. Plasticity is a fundamental characteristic of stem cells and it is regulated by reversible epigenetic modifications. Although gene-restriction programs are established during embryonic development when cell lineages are formed, stem cells retain a degree of flexibility that is essential for tissue regeneration. For instance, quiescent adult stem cells can be induced to proliferate and trans-differentiate in response to injury. The same degree of plasticity is observed in cancer, where cancer cells with stem cell characteristics (or cancer stem cells) are formed by transformation of normal stem cells or de-differentiation of somatic cells. Reprogramming experiments with normal somatic cells and cancer cells show that epigenetic landscapes are more plastic than originally thought and that their manipulation can induce changes in cell fate. Our knowledge of stem cell function is still limited and only by understanding the mechanisms regulating developmental potential together with the definition of epigenetic maps of normal and diseased tissues we can reveal the true extent of their plasticity. In return, the control of plastic epigenetic programs in stem cells will allow us to develop effective treatments for degenerative diseases and cancer.

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24.1 Introduction

How are cells in our body programmed to maintain their identity and function throughout life? The answer to this fundamental question is based on important processes that are initiated during embryo development and maintained in adulthood. This book chapter will describe and discuss the mechanisms that control cell and tissue homeostasis and how these are altered in cancer.

Cell identity is established during embryogenesis when the developmental potential of embryonic cells is restricted by differentiation programs that channel their fate to tissue-specific stem cells and specialised cell types. These dynamic events occur in cells with the same genetic information, thus cell fate depends on the epigenetic regulation of that genetic code. "Epigenetics" can be defined as regulation of gene expression that occurs by modifications imposed on the chromatin without change in the DNA sequence (Bird 2007). It is by changes in chromatin organisation that epigenetic modifications establish heritable transcriptional states responsible for the maintenance of cell function.

Epigenetic regulation includes DNA methylation, modification of histone tails and modulation by non coding RNAs (ncRNAs). Together with chromatin remodelling complexes, these modifications control chromatin organisation and regulate gene transcription (Jaenisch and Bird 2003).

DNA methylation is responsible for gene silencing and occurs at position 5 of cytosine (5mC) within CpG dinucleotides present in repetitive sequences and CpG islands in gene promoters and intragenic regions (Ball et al. 2009; Sharma et al. 2010). DNA methylation is maintained or established *de novo* by the DNA methyltransferases enzymes DNMT1 and DNMT3A/3B/3L, respectively (Bird 2002). Histone modifications comprise a vast range of post-translational modifications, such as acetylation, methylation, phosphorylation, ubiquitylation and ribosylation. These modifications can induce both activation and repression of transcription and their interactions function as a "code" defining cellular states (Turner 2007). Nucleosome remodelling and modulation by ncRNAs are the most important non covalent epigenetic modifications. Non coding RNAs, including microRNAs (miRNAs) and long non coding RNAs (lncRNAs), are single stranded transcripts involved in mRNA degradation and chromatin remodelling (Pauli et al. 2011). While heritable, epigenetic modifications are reversible and their dynamic interplay provides cells with ability to respond to environmental cues. Therefore it is easy to imagine how the epigenetic landscape created by these modifications can regulate phenotype plasticity in different cell types during normal development, but also cause disease if abnormally regulated.

Cancer is a disease characterised by abnormal cell proliferation and it is associated with both genetic lesions and epigenetic abnormalities. Because it can be portrayed as a process of aberrant cell proliferation and differentiation, cancer has been described as "a problem of developmental biology" where a marked resemblance between cancer cells and embryonic cells exists (Pierce and Johnson 1971). Indeed,

cancer cells re-initiate epigenetic programs that favour cell growth and survival at the expense of differentiation, thus behaving like undifferentiated embryonic cells and stem cells. As cancer cells depend on those mechanisms that maintain stem cell plasticity (Garraway and Sellers 2006), it is not a coincidence that many tumour suppressor genes that are epigenetically silenced in cancer are developmental genes involved in the regulation of stem cells (Barrero et al. 2010). It is precisely how stem cell plasticity is programmed in development and cancer that will be the focus of our discussion.

24.2 Epigenetics and Development

Epigenetic modifications regulate the acquisition of totipotency and subsequent progressive restriction of totipotent potential during embryonic development. Acquisition of totipotency is associated with two epigenetic reprogramming events: the formation of the zygote and the germ line (Hemberger et al. 2009). Both developmental stages require resetting of a differentiated epigenetic landscape to establish a new state with augmented developmental potency. Differentiation of somatic cells then requires the establishment of specific epigenetic programs that restrict their potential and maintain lineage memory. This section will describe the epigenetic modifications occurring during embryo development and explain how embryonic developmental potential is programmed in embryonic cells, somatic cells and germ cells.

24.2.1 Epigenetic Reprogramming During Embryo Development

Embryo development initiates with the fusion of the male and female pronuclei after fertilisation. The formation of the zygote is followed by epigenetic reprogramming of the specialised gametic genomes to ensure that the embryonic genome acquires totipotency, defined as the ability of a cell to form an entire organism. Immediately after fertilisation, the paternal nucleus undergoes profound chromatin remodelling. This involves exchange of protamines for histones in the nucleosomes and active DNA demethylation (Oswald et al. 2000; Mayer et al. 2000; Santos et al. 2002). Although a specific DNA demethylase enzyme has not been identified, a process involving DNA repair through the intermediate 5-hydroxymethylcytosine (5hmC) has been proposed (Wossidlo et al. 2010, 2011; Hemberger et al. 2009). After fusion, the progressive decline in DNA methylation up to the morula stage is due to passive loss of methylated cytosine

marks during DNA replication (Howell et al. 2001). Some genomic sequences escape this demetylation, including some repetitive sequences and most imprinted genes (Meissner 2010). Concurrent with DNA demethylation, reprogramming of histone modifications also takes place. The newly incorporated histones in the paternal pronucleus gradually increase active marks, such as acetylation of histone H3 at lysine 9 (H3K9ac), methylation of histone H3 at lysine 4 (H3K4me3), and repressive marks, e.g. methylated histone H3 at lysine 9 (H3K9me1, H3K9me2) and methylated histone H3 at lysine 27 (H3K27me3) (Meissner 2010). Subsequent to the first cleavage divisions, the embryo undergoes segregation of the first two lineages, the inner cell mass (ICM) and the trophectoderm. The cells of the ICM are pluripotent embryonic cells, able to differentiate to all somatic lineages and the germ line (Wray et al. 2010). Epigenetic programming of ICM cells includes *de novo* DNA methylation, acquisition of H3K9ac, H3K27me3, H3K4me3, H3K9me2 and H3K9me3 (Morgan et al. 2005). Re-establishment of DNA methylation is essential for normal embryonic development, as demonstrated by knockout experiments where deletion of DNMTs and other epigenetic modifiers participating in DNA methylation (LSH and G9a) causes embryonic lethality (Okano et al. 1999; Myant et al. 2011). During gastrulation and differentiation of embryonic cells to somatic lineages, a progressive decrease in plasticity is observed and this is accomplished by a program of epigenetic modifications that restricts cell fate, retains cell memory and confers cellular specialisation.

However, epigenetic restrictions imposed during differentiation are reprogrammed in the germ line. Germ cells derive from embryonic precursors of gametes defined as primordial germ cells (PGC), which are responsible for the development of a new organism in the next generation. Epigenetic reprogramming in the germ line is essential for the generation of a cellular state that will allow totipotency in the newly formed embryo. In addition, reprogramming of PGC ensures an equivalent epigenetic state in both sexes prior to differentiation into mature gametes and erasure of acquired epimutations which could be inherited in the next generation (Allegrucci et al. 2005). PGC are specified in the proximal epiblast and then migrate through the hindgut to the developing gonads. It is during migration and after colonisation of the gonads that extensive epigenetic reprogramming occurs in these cells. This involves loss of H3K9me2 and DNA methylation, and an increase in H3K27me3. It is thought that this epigenetic configuration, enriched in H3K27me3, H3K4me2/me3 and H3K9ac confers PGC with the required plasticity to regain pluripotency (Hemberger et al. 2009). In addition, loss of DNA methylation at imprinted genes ensures erasure of epimutations and correct re-establishment of monoallelic expression for gene dosage in the next generation (Allegrucci et al. 2005; Sasaki and Matsui 2008).

The epigenetic reprogramming and programming of cell plasticity during development is orchestrated by a battery of epigenetic modifiers. Their coordinated action ensures a correct program of cell proliferation and differentiation (Table 24.1).

Table 24.1 Key epigenetic modifiers regulating cell proliferation and differentiation

Epigenetic modifier	Modification/Function	Epigenetic mark	Implicated in cancer
EZH1/2	Histone methylation	H3K27me3	√
SUZ12	PcG-PRC2 complex	–	√
EED	PcG-PRC2 complex	–	√
JARID2	Recruits/stabilise PRC2	–	–
RING1	Histone ubiquitylation	H2AK119u	√
BMI-1	PcG-PRC1 complex	–	√
SET/MLL	Histone methylation	H3K4me3	√
	Histone acetylation	H4K16ac	√
UTX	Histone demethylation	H3K27me3	√
JMJD3	Histone demethylation	H3K27me3	√
JARID1A/B	Histone demethylation	H3K4me3	√
JMJD1A	Histone demethylation	H3K9me2	√
JMJD2C	Histone demethylation	H3K9me3	√
G9a	Histone methylation	H3K9me1/2	√
DNMT1	5mC-DNA methylation	5mC	√
DNMT3A	5mC-DNA methylation	5mC	√
DNMT3B	5mC-DNA methylation	5mC	√
DNMT3L	5mC-DNA methylation	5mC	√
GADD45	DNA demethylation	–	√
AID	DNA demethylation	–	–
LSD1	Histone demethylation	H3K9me2	√
		H3K4me1/2/3	√
SUV39	Histone methylation	H3K9me3	√
BRG1	Chromatin remodelling	–	√
BAF250	Chromatin remodelling	–	√
BAF155	Chromatin remodelling	–	√
CHD1/3/4/7	Chromatin remodelling	–	√
HDAC1/2	Histone deacetylation	–	√
MBD3	Chromatin remodelling	–	–
BPTF	Chromatin remodelling	–	√
TIP60-p400	Chromatin remodelling	–	√
CBP/p300	Histone acetylation	–	√
SETDB1	Histone methylation	H3K9me3	√
DICER	ncRNA processing	–	√
TET1/2/3	DNA hydroxymethylation	5hmC	√

24.3 Epigenetic Regulation of Stem Cells

In the previous section we have reviewed the epigenetic events that regulate development and program cell differentiation in the embryo. Although pluripotent cells exist only for a limited period of time before gastrulation, they can be isolated from the embryo and maintained *in vitro* as embryonic stem cells (ESC). Therefore ESC can be studied as *in vitro* model of naive embryonic cells and differentiated into

many different cell types. Differentiation is not limited to embryonic development, but it continues in the adult as continuous supply of specialised cells is needed for tissue turn-over and repair. This is accomplished by lineage restricted multipotent cells, or adult stem cells (ASC). Correct stem cell function is essential during an individual's life starting at the time when tissues are formed and later on, when they need to be regenerated and repaired. By analysing the epigenetic control of ESC and ASC, this section will describe how developmental plasticity of stem cells is programmed for correct function. Knowledge of how stem cell programs are regulated is important not only to advance stem cell-based therapies but also to understand how we can overcome diseases characteristic of stem cell dysfunction.

24.3.1 Control of Embryonic Stem Cells

ESC can be derived from the blastocyst ICM and their pluripotency maintained *in vitro* for many cell generations. ESC can symmetrically self-renew, hence giving rise to two identical stem cells. ESC ability to self-renew and to respond to developmental cues is controlled by a unique gene expression program. The ground state of pluripotent ESC is defined by the expression of a core network of transcription factors that include OCT4, SOX2 and NANOG. These factors act both as transcription activators and repressors, by activating genes involved in cell proliferation and self-renewal while repressing the expression of lineage-specific genes promoting differentiation (Young 2011). This bivalent state of ESC is essential for pluripotency and it is regulated epigenetically by the interplay of core transcription factors and Trithorax (TrxG) and Polycomb (PcG) epigenetic modifiers. TrxG-related proteins (SET/MLL) catalyse H3K4me3 at promoters of active genes, whereas PcG proteins catalyse histone modifications that are associated with gene silencing. PcG proteins include two complexes, PRC1 and PRC2, responsible for H3K27me3 and ubiquitylation of histone H2A at lysine 119 (H2AK119u), respectively (Meissner 2010). While PRC2 is required for initial gene silencing, recruitment of PRC1 stabilises the established transcriptionally repressive state. It is the presence of both active H3K4me3 and repressive H3K27me3 marks (bivalent domain) at developmentally regulated genes that allows ESC to remain in a poised state, ready for activation upon differentiation. Therefore ESC show a global open chromatin structure, with about 75% of gene promoters enriched for H3K4me3. These promoters can be active or inactive, depending on H3K27me3 co-occupancy.

Among silencing mechanisms DNA methylation plays a fundamental role in ESC. ESC present about 60–80% of methylated CpG nucleotides, with a unique distribution (Meissner 2010). Comprehensive maps of DNA methylation in ESC have demonstrated that the majority of high CpG promoters (HCP) are lacking methylation and are enriched in H3K4me3. These represent housekeeping genes, pluripotency genes and key developmental genes. In contrast, tissue specific gene promoters with low CpG density (LCP) are mostly methylated (Mikkelsen et al. 2007; Meissner et al. 2008). Therefore an epigenetic landscape presenting either

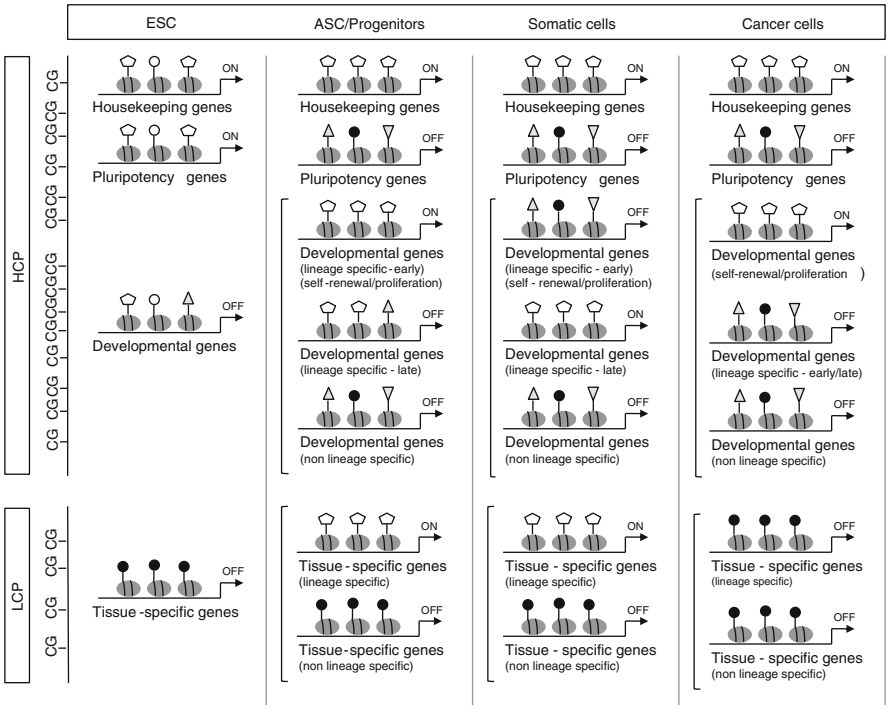

Fig. 24.1 Epigenetic landscapes regulating stem cell plasticity in development and cancer. HCP and LCP gene promoters are enriched for genes with different epigenetic regulation in different cell types. The figure shows how housekeeping genes, pluripotency genes, developmental genes and tissue-specific genes are epigenetically regulated in ESC, ASC, differentiated (somatic) cells and cancer cells. During differentiation there is a decrease in cell plasticity due to loss of bivalent domains and acquisition of repressive chromatin marks that restrict cell fate. Cancer cells reactivate an epigenetic landscape that is more plastic and shifted towards self-renewal and proliferation at the expense of differentiation (⌂: H3K4me3; ○: 5hmC; △: H3K27me3; ●: 5mC; ▽: H3K9me2/3)

unmethylated promoters (HCP with H3K4me3 or bivalent domain with H3K4me3/H3K27me3), or methylated promoters (LCP) define ESC (Fig. 24.1). In addition to methylation of CpG dinucleotides, other modifications of the DNA have been discovered in ESC. These include cytosine methylation in a non CG context (Lister et al. 2009) and cytosine hydromethylation (5hmC). Hydroxylation of 5mC to 5hmC is catalysed by the TET family of enzymes (Koh et al. 2011) and it is believed to be involved in the demethylation of 5mC and prevention of DNMTs activity (Xu et al. 2011). A genome-wide study of 5hmC in ESC revealed that this mark is enriched in gene bodies, transcription start sites of HCP promoters and enhancers. Bivalent or PcG only marked promoters are also particularly enriched for 5hmC (Xu et al. 2011; Pastor et al. 2011). Although a clear function for 5hmC in transcription regulation is still elusive, its distribution suggests a role in preparing genomic loci for activation upon differentiation. Indeed, 5hmC has been shown to be present in ESC, but

declines after differentiation (Tahiliani et al. 2009; Ficz et al. 2011). Finally, ncRNAs also participate in the epigenetic regulation of ESC plasticity. MicroRNAs regulate stability and translation of mRNAs involved in stem cell self-renewal and differentiation. ESC express a unique set of miRNA whose transcription is regulated by the core pluripotency factors, and these miRNA are involved both in sustaining self-renewal (e.g mir-290-295/302) and inducing rapid degradation of ESC transcription factors during differentiation (e.g mir-145) (Marson et al. 2008; Tay et al. 2008; Xu et al. 2009). In addition, many lineage-specific miRNA gene promoters are co-occupied by OCT4/NANOG/SOX2 and PcG and these are repressed in ESC, but become active upon differentiation (e.g let-7, mir-155, mir-124, mir-9) (Young 2011; Pauli et al. 2011). Balance between self-renewal and differentiation is a key characteristic of ESC. Altogether bivalent histone modifications, DNA methylation and miRNAs contribute to the establishment of an open chromatin state that allows undifferentiated cell function and the ability to respond to developmental signals in a timely fashion.

24.3.2 Control of Adult Stem Cells and Somatic Cells

Differentiation of pluripotent cells is associated with a loss of developmental potency that ensures cellular specialisation and committed identity. During lineage specification, ASC are formed and it is from these committed multipotent stem cells that specialised cells are derived. The role of stem cells in the adult is to maintain tissue homeostasis by regenerating aged or damaged cells. ASC reside in tissue-specific niches that control their asymmetrical self-renewal, defined as the ability to form a stem cell and a differentiated progenitor at cell division. ASC are more restricted in their differentiation potential compared to ESC as they can only give rise to multiple cell types within a tissue under physiological conditions. Genome-wide maps of epigenetic modifications in ASC and differentiated cells show that restriction in developmental potential is associated with a resolution of ESC open chromatin into a more restricted configuration (Fig. 24.1). Silencing of pluripotency genes is readily observed during differentiation, by loss of H3K4me3, gain of H3K27me3, H3K9me3, DNA methylation (Barrero et al. 2010) and expression of specific miRNAs (e.g. mir-134, mir-296, mir-470) (Tay et al. 2008). Differentiation into a specific lineage involves expression of genes specific to that cell type and silencing of genes expressed in other tissues. In this way, differentiation into the neural stem/progenitor cells (NSC) is accompanied by a decrease of H3K27me3 at neural genes silenced by bivalent marks, which correspond to increased gene expression. Genes poised or weakly induced retain bivalent marks, while H3K27me3 silencing is increased in non-neural lineage genes, together with H3K9me3 (Hawkins et al. 2010; Bernstein et al. 2006; Mikkelsen et al. 2007; Bracken et al. 2006). The same pattern is also observed in muscle and germ cell differentiation (Caretti et al. 2004; Chen et al. 2005; Asp et al. 2011), suggesting a PcG-mediated regulation of cell fate decisions.

Both H3K4me3 and bivalent HPC remain mostly unmethylated during differentiation. In contrast, resolution to univalent H3K27me3 mark results in an increase in DNA methylation and a complete loss of the bivalent marks results in DNA hypermethylation. A different epigenetic regulation is observed at LCP associated with tissue specific genes, as methylated LCP associated with neural genes gain H3K4me3 and non lineage specific genes retain DNA methylation (Meissner et al. 2008). Although overall DNA methylation levels are similar in pluripotent and differentiated cells, a small subset of genes displays tissue specific methylation. A recent study demonstrated 491 differentially methylated regions (DMR) being more methylated in fibroblasts compared to ESC (Lister et al. 2009), with DMR representing only 6–8% of CpG islands in different tissues (Berdasco and Esteller 2010). Important DNA methylation differences can be observed in ASC compared to differentiated cells of the same lineage. For instance, breast self-renewal and proliferation genes are hypomethylated in $CD44^{+}/CD24^{-}$ stem cells compared to differentiated luminal $CD24^{+}$ cells (Bloushtain-Qimron et al. 2008).

Many studies indicate DNA methylation as a mechanism providing long term gene silencing and epigenetic memory in differentiated cells, however experiments of conditional deletion of DNMT1 suggest that DNA methylation plays also an important role in maintaining ASC self-renewal and suppressing differentiation (Sen et al. 2010). Indeed, loss of methylation causes differentiation alterations in epithelial progenitor cells (EPC) (Sen et al. 2010) and hematopoietic stem cells (HSC) (Trowbridge et al. 2009; Broske et al. 2009). Other epigenetic mechanisms are involved in regulation ASC self-renewal, but their relation to DNA methylation is still unknown. For instance, the PCR1 PcG protein BMI-1 is required for NSC, HSC, mammary and intestinal stem cell proliferation (Molofsky et al. 2003; Lessard and Sauvageau 2003; Pietersen et al. 2008; Sangiorgi and Capecchi 2008), while overexpression of PCR2 PcG protein EZH2 blocks differentiation of myoblasts and EPC (Caretti et al. 2004; Sen 2011) and prevents HSC exhaustion (Kamminga et al. 2006). Because cell memory is set during development and inherent to each tissue-type, it was long assumed that differentiation of ASC is strictly specific to their lineage. However, recent studies demonstrate that under certain conditions, particularly after injury, ASC can trans-differentiate into cells of different tissues (Lotem and Sachs 2006). Therefore ASC, like ESC, show a differentiation plasticity that is conferred by epigenetic programs that can reversibly regulate transcription of genes expressed in different tissue according to physiological and pathological signals. The contribution of ASC plasticity to cancer will be described in the next section.

24.4 Cancer Stem Cells

The idea that cancer is caused by transformed cells with stem cell properties is not novel, but it has received renewed interest among scientists in recent years. The observation that tumours are formed by cells with functional heterogeneity has led to the postulation of two mutually exclusive models for the cellular origin of

cancer: the stochastic model and the cancer stem cell hierarchy. The stochastic (or clonal evolution) model predicts that every cell can become tumorigenic under the influence of endogenous (transcription factors) and exogenous (microenvironment) factors that can generate their own heterogeneous sub clones (Nowell 1976). In the stochastic model, cancer cells fluctuate between several states, owing to their plasticity (Wang and Dick 2005). In contrast, the cancer stem cell (CSC) model considers that tumours originate from transformed stem cells and they are organised in a hierarchical manner, whereby CSC lies at the apex and the proliferating progenitors and terminally differentiated cancer cells reside at the bottom of the hierarchy (Bonnet and Dick 1997). Recent studies show that both models can act together depending on microenvironmental signals and that tumour initiating cells can originate both from transformation of normal ASC or epigenetic reprogramming of more differentiated cells (Campbell and Polyak 2007) (Fig. 24.2). Both theories converge on the idea that cancer arises from transformed cells that acquire growth and survival advantage, which is a landmark of stem cells. The theory that cancer could arise from embryo-like cells was proposed about 150 years ago (Virchow 1855) and was later developed by Cohnheim and Durante with the concept of "maturation arrest", according to which cancer could develop from embryonic rudiments remaining in adult organs (Cohnheim 1867; Durante 1974). These theories were proven years later by studies on germ cell tumours demonstrating that teratocarcinomas contain CSC with very similar characteristics to ESC, with self-renewal and differentiation potential (Sell and Pierce 1994; Sperger et al. 2003). For a long time it was known that only a small population of cancer cells is tumorigenic and can propagate the tumour. Single-cell analysis of leukaemia revealed two different populations of cancer cells in terms of proliferative kinetics: the frequent large, fast-cycling cells and the rare, smaller slow-cycling cells with the same properties to that of normal HSC (Clarkson 1974). Through elegant studies, Dick and colleagues proved the existence of CSC by showing that in acute myeloid leukaemia (AML), a rare population of CSC with $CD34^{+}/CD38^{-}$ cell surface expression were able to recapitulate the original disease over repeated transplantation into NOD/SCID (non-obese diabetic/severe combined immunodeficiency) mice (Lapidot et al. 1994). Since then, CSC have been identified and isolated in solid tumours including breast (Al-Hajj et al. 2003), brain (Singh et al. 2003), melanoma (Fang et al. 2005), pancreatic (Hermann et al. 2007), prostate (Tang et al. 2007) and ovarian cancers (Bapat et al. 2005) (Table 24.2). CSC are a rare population of cells that resemble normal stem cells. They can self-renew, are long-lasting, remain relatively quiescent, and can generate all heterogeneous cell types comprising the tumour. CSC can lay dormant within their niche and therefore escape chemotherapy, which only targets highly proliferating cells. Their resistance to current cancer therapies (chemotherapy and radiotherapy) is also due to expression of ATP-binding cassette (ABC) transporters (pumping out harmful drugs), increased free radical scavenging and high expression of anti-apoptotic proteins (Visvader 2011). Since CSC retain many features of normal stem cells, their identification often relies on the expression of tissue specific stem cell markers. For example, leukemic stem cells can be

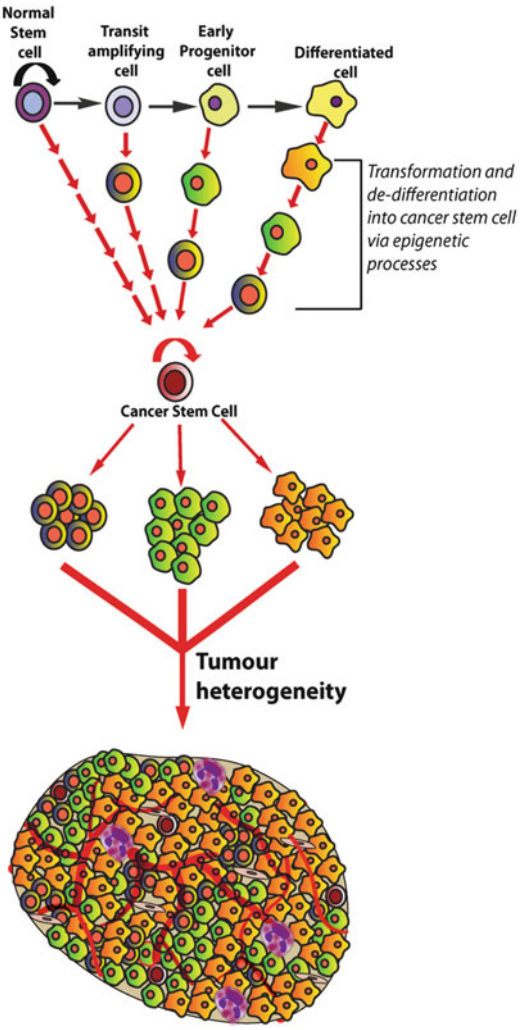

Fig. 24.2 Cancer stem cell-of-origin model. Different cell types from the lineage hierarchy can undergo oncogenic events to transform into tumour cells. CSC can either originate from a transformed normal stem cell, transit-amplifying cell, progenitor cell, or a terminally differentiated cell to give rise to a heterogeneous population of cancer cells

identified by CD34 and CD38 which are expressed on the cells in the HSC hierarchy (Bonnet and Dick 1997). Other universal CSC markers are instead based on their ability to pump out toxicants, which defines them as "side population" cells able to extrude a Hoechst dye, expressing ABC transporters and the detoxifying enzyme ALDH1 (Visvader and Lindeman 2008). Functional assays for CSC identification include xenografts into immunocompromised mice and formation of spheroids in culture. The xenograft assay involves transplanting cancer cells into NOD/SCID mice for tumour formation. Isolated CSC are generally more tumorigenic than differentiated tumour cells and their serial transplantation shows that they can reproduce the original disease through every passage. Sphere forming assays, which involve culturing CSC under stem cell conditions, preserve survival

Table 24.2 CSC markers in human tumours

Cancer	CSC markers	Cell-of-origin	References
Acute myeloid leukemia (AML)	$CD34^+CD38^-$	HSC	Bonnet and Dick (1997)
	ALDH1		Ran et al. (2009)
Acute lymphoblastic leukemia (ALL)	$CD34^+CD19^-CD10^-$	HSC	Cox et al. (2004)
Breast	$CD44^+CD24^{low/-}$	Mammary stem cells	Al-Hajj et al. (2003)
	ALDH1		Ginestier et al. (2007)
Colon	$CD133^+$	Intestinal stem cells	O'Brien et al. (2007)
	$EpCAM^{hi}$ $CD44^+$		Dalerba et al. (2007)
Melanoma	JARID1B	Skin stem cell	Roesch et al. (2010)
	$ABCB5^+$		Schatton et al. (2008)
Prostate	$CD133^+$ TRA-160	Basal progenitor cells	Goldstein et al. (2010)
	$TRA\text{-}160^+CD151^+CD166^+$		Rajasekhar et al. (2011)
Pancreas	$CD24^+CD44^+EpCAM^+$	_	Li et al. (2007)
Brain	$CD133^+$	NSC	Singh et al. (2003)
Head and neck	$CD44^+$	_	Prince et al. (2007)
Lung	$CD133^+$	Bronchioalveolar stem cells	Eramo et al. (2008)
	ALDH1		Jiang et al. (2009)
Liver	$CD44^+$ $CD90^+$	_	Yang et al. (2008)
Ovary	$CD44^+$ $CD177^+$	_	Zhang et al. (2008)
Stomach	$CD44^+$	_	Takaishi et al. (2009)
Osteosarcoma	$CD133^+$	Mesenchymal stem cells	Tirino et al. (2008)

of CSC while inducing cell death by apoptosis in non-CSC (Visvader and Lindeman 2008). However, regardless of the assay, a major challenge for studying CSC is their inherent developmental plasticity, which involves the co-existence of different epigenetic states during cancer progression. For instance, $CD44^+/CD24^-$ breast CSC exist in a metastable state oscillating between differentiation and de-differentiation, with CSC giving rise to luminal $CD24^+$ cells and luminal cells de-differentiating back into CSC (Meyer et al. 2009). The same has been observed in melanoma, where $JARID1B^+$ CSC generate $JARID1B^-$ cells and vice versa (Roesch et al. 2010). In addition, CSC plasticity can often extend beyond their lineage and they can express genes normally expressed in different tissues. Consistent with the trans-differentiation potential of ASC after injury, CSC show the same plasticity resulting in abnormal tissue regeneration (Lotem and Sachs 2006). CSC plasticity is influenced by embryonic developmental programmes and a similar gene expression signature between highly malignant, poorly differentiated solid tumours and ESC has been reported (Ben-Porath et al. 2008). This is due to the ability of cancer to take control of normal developmental programs for selective advantage, albeit in part related to an upregulated Myc-regulatory network (Kim et al. 2010). For example, the epithelial-to-mesenchymal (EMT) transition, a reversible embryonic programme that allows transition between cellular phenotypes during gastrulation, contributes to CSC plasticity. EMT is recapitulated during

tumour progression and metastasis by a transition from an epithelial to a mesenchymal phenotype with acquired cell motility. This is induced by activation of key signalling pathways (TGF-β, Notch, FGF) that drive epigenetic silencing of the adhesion molecule E-cadherin (Thiery et al. 2009). EMT is also important for maintenance of stem cell properties and CSC can hijack this program to regulate their plasticity. In addition to this, CSC establish their own niche by recruiting cells to recreate a similar microenvironment to that of a normal stem cell niche. The niche can induce and expand CSC by enhancing "stemness" features in non tumourigenic cells by overexpressing signals that are important for stem cell renewal and promote EMT through epigenetic alterations (Mani et al. 2008).

24.4.1 Epigenetic Origin of Cancer Stem Cells

Epigenetic alterations are generally observed at early stages of tumorigenesis and are likely candidates for a mechanism of tumour initiation. ASC are long-lived and during their aging process they may undergo epigenetic insult which can induce survival programs and predispose to the onset of cancer after further genetic and epigenetic alterations (Feinberg et al. 2006; Baylin and Ohm 2006). Numerous evidences indicate a role for epigenetic defects in the development of CSC. Normal stem cells are vulnerable to epigenetic alteration when induced to sustained self-renewal. Extensive DNA methylation alterations have been reported in ESC after long term in culture, with changes which are inherited after differentiation and associated with cancer (Allegrucci et al. 2007). Similar alterations have been observed in NSC (Shen et al. 2006), with a recent study demonstrating hypermethylation of HPC after many generations and inherited after differentiation to astrocytes (Meissner et al. 2008). Hypermethylation of bivalent domain genes in stem cells is particularly important for tumorigenesis as tumour suppressor genes have bivalent promoters in ESC and ASC (Barrero et al. 2010) and hypermethylation of tumour suppressor genes is a hallmark of cancer (Jones and Baylin 2007). As bivalent genes are developmental genes and transcription factors that regulate stem cell fate, it seems apparent how their epigenetic silencing in ASC could generate stem cells locked in a self-renewal state with impaired or limited differentiation potential (Fig. 24.1). Several studies have demonstrated that PcG target genes are much more likely to become hypermethylated in cancer (Widschwendter et al. 2007; Ohm et al. 2007; Schlesinger et al. 2007) and a mechanism by which the PcG-H3K27me3 mark could direct DNA methylation has been proposed (Keshet et al. 2006; Vire et al. 2006). In addition, overexpression of PcG proteins BMI-1 and EZH2 are also often found in cancer and they both play a fundamental role in regulating stem cell function (Bracken and Helin 2009). DNA methylation alterations at other genomic regions can also participate in the development of CSC. Hypomethylation of the genome can induce chromosome instability together with aberrant activation of proto-oncogenes associated with stem cell self-renewal and proliferation (Sharma et al. 2010). Chromosome translocations producing MLL fusion proteins are involved in AML, with more than

50 different fusion partners being identified. Importantly, MLL-ENF fusion is able to transform HSC and committed progenitors, thus creating CSC with acquired self-renewal and de-differentiated phenotype (Milne et al. 2005).

Loss of imprinting (LOI) via DNA demethylation can also be associated with growth advantage in stem cells and biallelic expression of IGF2 accounts for half of Wilms tumours and predisposition to colon cancer. Other LOI involved in cancer include PEG1/MEST involved in lung cancer, CDK1C in pancreatic cancer, TP73 in gastric cancer and DIRAS3 in breast cancer (Feinberg et al. 2006). Finally, DNA methylation at promoters of miRNA genes involved in stem cell differentiation can lead to CSC. For instance, silencing of the miRNA *let-7* contributes to breast, colon and lung cancer (Zimmerman and Wu 2011) and silencing of the mir-200 gene family induces EMT and CSC phenotype in breast, lung and ovarian cancer (Brabletz and Brabletz 2010). As epigenetic alterations can result in CSC and plasticity is a fundamental property of stem cells, we should not be surprised that CSC share this characteristic. What becomes apparent is that the effect of the environment is of primary importance for cancer initiation and progression while the behaviour of stem cells is completely dependent on physiological and pathological signals. As controlling environmental conditions is not an easy endeavour, we need to develop treatments that are able to completely eradicate CSC as their plasticity is a likely prospect of tumour recurrence.

24.5 Resetting Cancer by Epigenetic Re-programming

The developmental plasticity demonstrated by CSC and the reversible nature of epigenetic alterations has led to the development of epigenetic therapies as a new treatment option for cancer patients. Several epigenetic drugs that aim at halting tumour progression and restoring normal cell function are being tested in human clinical trials. Because epigenetic drugs can induce differentiation of ESC and ASC, their use for resumption of normal tissue differentiation is also been tested (Berdasco and Esteller 2010). The concept of differentiation therapy as an alternative or adjuvant treatment to chemotherapy has been inspired by many years of research. Landmark experiments have shown that embryonic microenvironments that program cell fate during development are able to reverse malignancy by resetting normal pathways of cell differentiation. For instance, teratocarcinoma cells can be induced to differentiate when transplanted into chimeric embryos, and malignancy can be reverted by injecting cancer cells into zebrafish, chicken and mouse embryos (Telerman and Amson 2009). Nuclear transfer experiments have shown that the epigenotype of cancer cells can be reprogrammed by oocyte molecules (Blelloch et al. 2004; Hochedlinger et al. 2004) and recent experiments with oocyte extracts have shown that this effect is mediated by chromatin remodelling and reactivation of silenced tumour suppressor genes resulting in reduction of tumour growth in mouse xenografts (Allegrucci et al. 2011). New insights have also come from studies of reprogramming to pluripotency. Yamanaka's work demonstrated that the ectopic expression

of core pluripotency factors (OCT4, SOX2, MYC, KLF4) in somatic cells can reprogram the epigenetic state of somatic cells to that of pluripotent cells giving rise to induced pluripotent stem cells (iPSC) (Takahashi and Yamanaka 2006). With a similar approach, cancer cells are reprogrammed to induced pluripotent cancer cells (iPC) with re-acquired differentiation potential (Sun and Liu 2011). Reprogramming studies suggest that even if finely regulated, epigenetic landscapes are plastic and reversible and that cellular transformation and de-differentiation share similar epigenetic mechanisms. This view is sustained by the evidence that many factors able to reprogram somatic/cancer cells to induced pluripotent cells act as oncogenes. This is the case for OCT4 and SOX2 (aberrant expression is tumorigenic in epithelial tissues), NANOG (overexpressed in germ cell tumours), KLF4 (associated with colorectal cancer), LIN28 (associated with hepatic cancer) and MYC (potent oncogene involved in many cancer types) (Daley 2008). Therefore, differentiation therapy could be the way to reset CSC to normal function and epigenetic programs that regulate stem cells are of particular interest as novel treatment targets.

24.6 Conclusions

More than 50 years have passed since Waddington proposed the idea that signals from embryonic environments can influence cell fate and behaviour according to an "epigenetic landscape" (Waddington 1940). He proposed that embryonic development can be visualised as a landscape delimited by hills and valley, where cells can take different directions depending on the signals received along the path. However, possible paths in this landscape are restricted by barriers dictated by hills and only defined valleys are available when cells are restricted to a defined initial trajectory. With a modern view, we identify these landscapes as epigenetic modifications regulating expression of developmental genes during differentiation in a coordinated fashion. Although cell fate is developmentally established, a degree of plasticity is retained for tissue turnover or acquired in pathological conditions. Waddington recognised that cancer cells escape the effect of developmental forces (Waddington 1935), introducing the concept of cancer as a defect in the mechanisms that control cell differentiation. We now know that epigenetic alterations are hallmark of cancer and they can transform normal cells to tumour cells with altered proliferation and differentiation. Our knowledge of cell plasticity has greatly increased over the last few years as stem cell technologies have developed and genome-wide mapping of epigenetic modifications of stem cells and somatic cells are being established. Defining the epigenome of cellular states in normal and cancer tissues is therefore a new challenge, but a most beneficial one as it holds the promise to eradicate or control cancer, an expected disease of an aging population.

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Chapter 25
Histone Acetylation as a Therapeutic Target

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Abstract The recent developments in the field of epigenetics have changed the way the covalent modifications were perceived from mere chemical tags to important biological recruiting platforms as well as decisive factors in the process of transcriptional regulation and gene expression. Over the years, the parallel investigations in the area of epigenetics and disease have also shown the significance of the epigenetic modifications as important regulatory nodes that exhibit dysfunction in disease states. In the present scenario where epigenetic therapy is also being considered at par with the conventional therapeutic strategies, this article reviews the role of histone acetylation as an epigenetic mark involved in different biological processes associated with normal as well as abnormal gene expression states, modulation of this acetylation by small molecules and warrants the possibility of acetylation as a therapeutic target.

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25.1 Histone Acetylation in Physiology

25.1.1 Introduction

Epigenetics 'is the structural adaptation of chromosomal regions so as to register, signal or perpetuate altered activity states' (Bird 2007), which results in altered gene expression through the mechanism(s) other than mutation in the underlying DNA sequences. Most of these epigenetic modifications are reversible, therefore not inherited. Some epigenetic modifications like selective histone methylation, methylated DNA regions and altered chromatin structures could be inherited across several cycles of cell division. Both histones and nonhistone proteins undergo epigenetic alterations through post-translational modifications. Several post-translational modifications (PTMs) like acetylation, methylation, phosphorylation, parylation, ubiquitination, sumoylation, acylation etc., ornate the histones in a context dependent manner and in turn regulate various biological processes.

25.1.2 Acetylation and Deacetylation

Protein lysine acetylation is one of the key regulators of biological functions of histones and several nonhistone proteins. Protein acetylation is brought about by the transfer of acetyl group from acetyl coenzyme A (acetyl CoA) to lysine residues. This reaction is catalyzed by a group of proteins called lysine/histone acetyltransferases (KATs/HATs). Based on their cellular localization KATs are classified into nuclear or type A and cytoplasmic or type B KATs. There are only three cytosolic KATs: HAT1 (KAT1), HAT2 and HAT4 (Blackwell et al. 2007; Chang et al. 1997; Takahashi et al. 2006) reported till date and they acetylate nascent histones. Nuclear KATs are further classified into five families based on their structural and functional differences (Fig. 25.1). There are three members of GNAT family- Gcn5 (KAT2A), p300/CBP associated factor (PCAF/KAT2B) and ELP3 (KAT9). Gcn5 and PCAF acetylate both histone and nonhistone substrates and they are part of various complexes involved in diverse physiological functions (Nagy and Tora 2007). There are two homologs in p300/CBP family- p300 (KAT3B) and CREB binding protein (CBP/KAT3A). Both of them are transcription coactivators and they have overlapping as well as distinct biological roles (Kalkhoven 2004). Tip60 (KAT5), MOZ (KAT6A), MOF (KAT8), MORF (KAT6B) and HBO1 (KAT7) are the major members of MYST family of KATs and they play crucial role in DNA damage repair, development and differentiation (Sapountzi and Côté 2011). There are few transcription factors- TFIIIC90 (KAT12), ATF2 and TAF1 (KAT4) that have inherent KAT activity and affect transcription directly. There are few nuclear hormone related KATs like SRC1 (KAT13A) and ACTR (KAT13B), which also act as coactivators. Though they possess histone acetylation activity, they are often part of p300/CBP

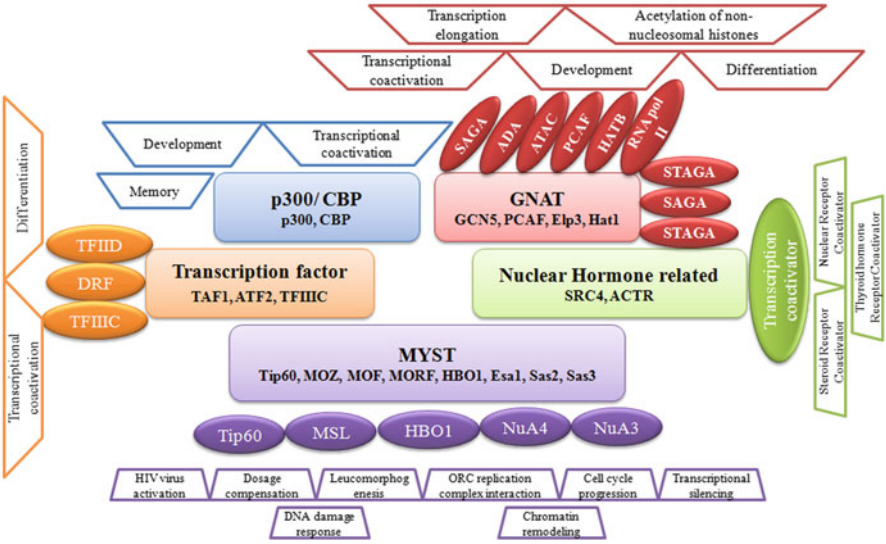

Fig. 25.1 KATs and their physiological roles. Different classes of KATs are involved in specific or overlapping cellular physiology. GNAT family of KATs are present in multiprotein complexes which are designated for different functions ranging from transcription regulation to differentiation. *Rectangular box*: KAT family, *Ovals*: Known complexes, *Trapezoids*: Biological functions

mediated coactivator complexes. Apart from these families there are few KATs: CIITA, CDYL, which does not fall under any family. The reversible acetylation marks on histones are removed by lysine deacetylases (HDACs/KDACs), which is essential for gene silencing. There are three major classes of KDACs (Hildmann et al. 2007). Class I KDACs (KDAC 1, 2, 3 and 8) are nuclear and involved in epigenetic regulations. They are always part of multienzyme complexes. Class-II KDACs (KDAC 4, 5, 6, 7) are characterized by nucleo-cytoplasmic shuttling. They can function both independently as well as part of a complex and are commonly involved in differentiation. NAD dependent KDACs are grouped under class III and they are generally called sirtuins. Sirtuins are involved in both transcription and metabolism.

25.1.3 Role of Histone Acetylation in Various Biological Processes

Specific histone acetylation is prerequisite for many biological processes starting from nuclear shuttling of histones, chromatin organization, replication, to gene expression through transcription activation. Histone acetylation/deacetylation is controlled by various external and internal signalling factors like hormones, growth factors, pathogen interactions, redox/metabolic state of the cells etc.

25.1.3.1 Replication

Replication in eukaryotic cells includes faithful propagation of DNA sequence as well as chromatin state at the different regions of the genome. Replication begins with decompaction of chromatin which allows replication machinery to bind to the replication origin (Falbo and Shen 2006). 'Replication fork' progresses via disruption of nucleosome in the front and transfer of the histones on to the leading or lagging strands. Finally, newly synthesized histones are incorporated during the replication dependent de-novo nucleosome assembly and daughter strands are produced (Groth et al. 2007a, b). The Type B acetyltransferases are predominantly associated with the replication function of acetylation. Newly formed free histone H4 is diacetylated at K5 and K12 by Hat1p/Hat2p complex, which helps in nuclear shuttling and nucleosome incorporation, but these marks are rapidly lost after completion of replication (Parthun et al. 1996; Taddei et al. 1999). Nuclear Hat1p complex is associated with acetylated H4 and H3 (K14 and K23) to help in H3-H4 tetramer assembly (Qin and Parthun 2002). The role of H4K16 and K8 in replication is yet to be ascertained. Further it has been shown that Hat1-RbAp46 complex, which acetylates H4, is essential for H3-H4 predeposition complex formation (Barman et al. 2008, 2006). The exact role of H4K5/K12 diacetylation during replication is yet to be completely understood. Recently, H4K16 acetylation was implicated in the recruitment of mini-chromosome maintenance (MCM) complex during replication initiation (Chiani et al. 2006). HAT1p/HAT2p sub complex is also part of origin recognition complex (ORC) during DNA replication in yeast (Suter et al. 2007). In yeast *Saccharomyces cerevisiae*, upon accumulation of replication stress or S-phase dependent double strand break, there is enrichment of H3K56 acetylation at the break site, indicating a significant role of H3K56 acetylation during replication (Vempati 2012; Vempati et al. 2010). Rtt109 mediated acetylation of H3K56 occurs in the newly synthesized H3-H4 dimer associated with Asf1 complex (Driscoll et al. 2007; English et al. 2006; Han et al. 2007). Recent report suggests that golgi apparatus localized HAT4 specifically acetylates free H4 at K20 (tail) and at K79 and K91 in the globular domain, thus involved in nucleosome assembly (Yang et al. 2011). Other chromatin modifications such as phosphorylation, methylation also cooperates in establishing the network associated with acetylation for the process of replication.

25.1.3.2 Repair

DNA double strand break (DSB) is constantly induced by both endogenous (metabolic) and exogenous (environmental) agents, which invokes an efficient mechanism of repair to maintain genomic stability. Growing body of evidence suggests that both homologous recombination and nonhomologous end-joining pathways are involved in DSB repair, which is further controlled by involvement of several PTMs. DSB induces instantaneous hypoacetylation of H3K9 and H3K56 by SIRT2 and SIRT3 (Tjeertes et al. 2009; Vempati et al. 2010). DSB also induces accumulation of Ku-70 and subsequent repair pathways (Krishnan et al. 2011). Post DSB repair, Nuclear NuB4 (Hat1p/Hat2p- Hif1p/Asf1) complex is involved in acetylated H3-H4

deposition and chromatin reassembly (Ge et al. 2011). Histone chaperone, anti-silencing function-1A (ASF1A), is crucial for post-repair H3K56Ac restoration, which in turn, is needed for the dephosphorylation of γ-H2AX and cellular recovery from checkpoint arrest. Complete restoration of H3K56Ac is monitored by ataxia telangiectasia mutated (ATM) checkpoint kinase (Battu et al. 2011). Tip60 and Mof mediated acetylation of H2AK5 and H4(K5/8/12/16) are also crucial for genomic stability after DSB repair. Glycogen synthase kinase-3 (GSK-3) mediated phosphorylation of Tip60 S86 is crucial for its activation (Charvet et al. 2011), which in turn acetylates p53 and H4 ultimately regulating the expression of PUMA (p53-upregulated mediator of apoptosis). Thus activation of p53 by DNA damage results in cell-cycle arrest, allowing DNA repair and cell survival, or induction of apoptosis. Apart from acetylation, other PTMs like protein methylation (Lake and Bedford 2007), phosphorylation (Charvet et al. 2011), ubiquitination (Wu et al. 2011), ADP-ribosylation (Messner and Hottiger 2011), sumoylation (Goodarzi et al. 2011) play crucial role in DNA DSB repair. The interplay between different PTMs and remodelling factors during repair have been discussed in details in the reviews by van Attikum and Gasser 2009; Lee et al. 2010.

25.1.3.3 Chromatin Organization

Acetylation at the lysine residues on histones leads to charge neutralization, which in turn reduces histone-DNA interaction leading to unwinding of the chromatin. This process is further assisted by ATP dependent remodelling complexes and is a prerequisite for all chromatin dependent phenomena. Histone acetylation in conjunction with other PTMs acts as the mark for site specific recruitment of various complexes and also contributes towards the processivity of the complex. Histone deacetylation precedes repression of active chromatin dependent processes and leads to chromatin compaction and heterochromatinization. Mechanistically, this process is a cascade of several events that include DNA methylation, histone methylation as well as histone deacetylation. These set of modifications are recognized by the repressor modules which further facilitate the compaction of the chromatin. The large scale reorganization is brought about by various chromatin associated proteins as well as the boundary elements that define the regions of transcriptional competence. Thus, acetylation is a very important mark essential for the overall structural organization of chromatin (Fukuda et al. 2006; Wilson and Merkenschlager 2006; Zhu and Wani 2010).

25.1.3.4 Transcription- RNA pol I/II/III

Histone tail acetylation at the gene promoters triggers a set of events that finally lead to the process of transcription. Along with ATP dependent remodelling complexes, H3K9 and K14 acetylation and H3K4Me3 help in recruitment of activator proteins at the promoter sites. This leads to recruitment of general transcription factors followed by the RNA polymerases to start the transcription. The H3K36 methylation,

H3 acetylation and other PTMs also play critical roles in transcription elongation and termination. The roles of epigenetic marks in the process of transcriptional regulation have been reviewed in details in part III of this book.

Histone Acetylation Associated Crosstalk During Transcription and Gene Expression

The histone acetylation associated crosstalk with the phosphorylation and methylation has been well documented in transcriptional regulation. A classical example is the phosphorylation of histone H3S10 which facilitates acetylation of H3K14 and thus activates transcription (Lo et al. 2000), which is further augmented by inhibition of HP1 recruitment at methylated H3K9 (Fischle et al. 2005). Bimodal function of methylated H3K4 is typified by recruitment of NuA3 histone acetyltransferase complex (Martin et al. 2006) and also a histone deacetylase complex (Shi et al. 2006), which shows context dependence of the crosstalks. Since the modification sites for acetylation and sumoylation (another post translational modification) have a considerable overlap, they also happen to reciprocally regulates each other's function (Chupreta et al. 2005). Transient loss of sumoylation leads to increase of acetylation and concurrent switch from a repressed to activated state in mammalian cells in a carbon source dependent manner (Segré and Chiocca 2011). Similar reciprocal role of acetylation and sumoylation has been observed during the regulation of ETS-domain transcription factor PEA3 (Guo et al. 2011) and oncoprotein Krüppel-like factor-8 (KLF8) (Urvalek et al. 2011). Histone H3K18 acetylation by p300/CBP incidentally acts as a mark for the subsequent arginine methylation by CARM1 at histone H3R17. Thus, the acetylation modification also influences the process of arginine methylation and has been identified as a part of the p53 dependent transcriptional activation cassette (An et al. 2004). Recently, it has been identified that acetylation also influences gene expression indirectly by modulating the miRNA expression. This evidence has been obtained by using the deacetylase inhibitors; a more detailed investigation is however needed with respect to the role of acetylation and miRNA expression. The integration of miRNA in the acetylation centred crosstalk, during transcription regulation is yet to be established.

Recent studies have further added another level of complexity by showing the spatio-temporal regulation of the PTMs. Zippo et al. have reported recruitment of oncogenic kinase PIM1 phosphorylates H3S10 at the enhancer region of serum responsive gene *FOSL1*, which then recruits 14-3-3 protein (Zippo et al. 2009). 14-3-3 complex recruits the KAT- MOF and brings about acetylation of H4K16 at the enhancer. Acetylated H4K16 recruits Brd4 and associated kinase P-TEFb, which phosphorylates RNA pol II and facilitates transcription elongation of *FOSL1* gene. This order of recruitment of phosphorylated H3S10 and acetylated H4K16 are in contrast to the previous report (Wang et al. 2001). These observations along with many others have shown that the order of recruitment of PTMs and their readers and writers decide the functional outcome (Lee et al. 2010; Smith and Shilatifard 2010).

25.1.3.5 Development and Differentiation

Histone acetylation plays crucial role in the different developmental stages and the epigenetic landscape during these stages are very different from that in the differentiated cells. Chromatin is in a more 'fluid' state in stem cells and also during the earlier stages of development, which helps in easy accessibility of the genetic information. The process of differentiation has been most well studied in the mouse embryonic stem cell model as well as lineage specific models such as myocyte, adipocyte, neuronal lineages etc. With respect to the developmental stages, the maximum information is from the mouse model. Several studies have also been done with the drosophila model such as the role of polycomb complexes. However, we shall be focusing on the stem cell based data available to review the status of chromatin and its functional modulation during the process of differentiation and development.

Chromatin During the Process of Differentiation and Development

Differentiation generally refers to the formation of functionally mature cell types. In this process there are stages of developmental timelines, wherein the cell attains partial functionality. In general, most of the adult stem cells belong to this category. The totipotent cell in the mammalian life cycle is restricted to the embryo till its cleavage stage. Following this, the cells attain a pluripotent state which signifies their ability to lead to the formation of the three germ layers (ectoderm, endoderm and mesoderm), from wherein, finally the more differentiated cell types gets established. These differentiated cell types also exist either as multipotent (hematopoietic lineage) or the unipotent (neuronal lineage). However, the terminally differentiated cell types are functionally complete. The chromatin, which is the nucleoprotein ensemble of the genetic material, is generally considered to be more fluidic, or in an open state. As the stages of differentiation proceeds, compaction of chromatin proceeds. The electron spectroscopic imaging studies reveals that the structural organization of ES cell chromatin is much more open and mostly more active transcriptionally (Fussner et al. 2010). This has also been validated by the studies looking for heterochromatin proteins whose reorganization has indeed validated this hypothesis that during the process of differentiation the chromatin does undergo a reorganization from an open chromatin state to an inactive, more compact form (Bártová et al. 2008).

Histone Acetylation During the Process of Differentiation and Development

The process of development and differentiation are events which are regulated by distinct transcriptional states and hence the role of acetylation in these processes is crucial. However, there exists stage specificity with respect to the expression profiles of these acetyltransferases and deacetylases. The possible role of acetyltransferases in the process of early development has been obtained from studies on the mouse model knockout systems. For a long time there were speculations that

such knockouts could be lethal since the acetyltransferases are widely considered to be redundant and absolutely essential for the transcriptional events. In contrary, till date distinct roles of acetyltransferases have been observed during development. More importantly, knockout of some KATs were not lethal and were shown to be critical for key developmental events. The lysine acetyltransferase family PCAF/GCN5 have distinct developmental roles. PCAF is expressed at later stages in the mouse development around E12 and hence does not show any phenotype on knockdown. GCN5, which is expressed at very early stages such as E7.5 replaces the PCAF, which is essential for the developmental processes since in the PCAF knockout, increased GCN5 levels have been observed in the liver and lungs (Yamauchi et al. 2000). But, the reverse was not observed when GCN5 was not present in which case the mice died in utero. The double knockout exhibited defects in vascular formation and few showed defects in the neural tube closure. However, it is clear that the expression of GCN5 and PCAF are timed distinctly and hence has potential distinct, and overlapping roles. The other important acetyltransferase, p300 and CBP were so difficult to be knocked out, that for several years their exact roles in the developmental stages could not be identified. Later on advancement in the technology of silencing in a specific manner, led to the specific knockdown at different stages as well as in different tissues. The most important information gained from these studies is that, probably p300 and CBP functions are redundant since one replaces the other (Goodman and Smolik 2000). However, in the neuronal developmental pathway the requirement of CBP has been shown to be absolutely critical. Subsequently, it was also identified that several neurodegenerative disorders were characterised by a loss in CBP acetyltransferase function further strengthening its need in neuronal processes. Recently, it has been very elegantly shown in tissue specific targeted knockouts that CBP acetyltransferase has a role to play in memory formation (Barrett et al. 2011). An independent study also investigated the role of p300 and GCN5 by knocking down both the acetyltransferases (Phan et al. 2005). This study led to the identification of critical roles of these enzymes in the early developmental processes. The MYST family acetyltransferases are also found to be essential for important developmental processes (Voss and Thomas 2009). The MOZ acetyltransferase has been shown to be necessary for the process of hematopoiesis in the mice (Katsumoto et al. 2006). The major hematopoietic organs were affected in this tissue specific knockdown. The Tip60 acetyltransferase has been essentially proven to have a tumor suppressor function. The homozygous knockdown was not viable and was defective in implantation. Therefore, heterozygous knockdown were generated which showed a haploinsufficiency with respect to tumor formation (Gorrini et al. 2007). Most of the acetyltransferases and deacetylases show differential expression levels during the process of early development. Subsequent to this, these enzymes become critical to several processes in gene expression. Hence, their expressions are more or less same during the later stages of development. Their activities do differ, thus the modulation during the process of differentiation is more at the activity level than the expression level itself. For example, the acetyltransferase PCAF which is expressed after E12.5 in mouse is present all through the stages of the myogenic differentiation process. However, the initiation of this process is

signified by the PCAF mediated MyoD acetylation (Described in details in Chap. 7 by Bharathy et al. of this collection). The process of differentiation is generally functional either during the later stages of development giving rise to the fully functional cell types or during diseases such as cancer. However, both these phenomena are characterized by almost similar mechanisms. The following section highlights the role of acetylation in different diseases including cancer.

25.2 Histone Acetylation and Diseases

The acetylation of histones and non histone proteins plays an important role in the various aspects of gene expression as discussed in the previous section. These key players also have a significant role to play in the process of disease manifestation and progression which is majorly due to a loss in the acetylation/deacetylation balance. This could be either because of the changes, in their activities or due to a change in their expression itself. The exact mechanism with respect to each of the KAT families, in disease manifestation and progression will be discussed in the next section. Of the different KAT families, we shall majorly focus on the CBP/p300 family, PCAF/GCN5 family and the MYST family.

25.2.1 Involvement of p300/CBP Family in Diseases

The p300/CBP family of KATs is represented by the p300 and CBP acetyltransferases. These are important transcriptional coactivators and are involved in several aspects of gene expression. Their involvement in certain diseases such as cancer, virus associated diseases, diabetes, cardiac hypertrophy and neurodegenerative disorders have been extensively investigated as discussed in the subsequent section.

25.2.1.1 Cancer

Role of p300 and CBP in cancer is highly controversial. There are reports suggesting role of p300 as a tumor suppressor whereas on the contrary p300 is also found to be a tumor promoter. It is also been reported that inhibition of p300 inhibits cancer cell growth, hence strengthening its role as a tumor promoter gene. Down regulation of p300 activity induces senescence and growth inhibition in melanocytes (Bandyopadhyay et al. 2002). In acute monocytic leukemia, MOZ translocates from chromosome 8 to chromosome 22 [t(8;22)(p11;q13)] where it forms a fusion protein with p300 (Kitabayashi et al. 2001). In acute myeloid leukemia, due to chromosomal translocation CBP forms a fusion protein with MOZ (Troke et al. 2006). These fusion proteins are more active in their function and cause aberrant hyperacetylation and transcriptional activation. Expression levels of p300 are found

to be very high in resectable esophageal squamous cell carcinoma (ESCC) than in normal esophageal mucosa cells. High p300 expression levels are also associated with higher grades of ESCC (Li et al. 2011b). Recent reports suggest that p300 mRNA and protein levels are very high in patients of hepatocellular carcinoma (HCC) and high expression of p300 is essential for acquisition of the aggressive phenotype (Li et al. 2011a). Studies from our group have also revealed the mechanistic link between the acetyltransferase p300 and the histone chaperone, NPM1 in the manifestation of oral cancer (Shandilya et al. 2009; Arif et al. 2010). p300 is an activator of Glial Fibrillary Acidic Protein *GFAP* gene which is associated with astrocytic differentiation of glioblastoma multiforme (GBM) cells. Silencing of p300 gene by RNAi induces invasion potential of (GBM) cells *in vivo* (Panicker et al. 2010). Histone H3K56 is a common site of acetylation for p300/CBP and is associated with progression of few cancers (Das et al. 2009).

Tumor suppressor function of p300 and CBP is supported by the observation that the monoallelic mutation of CBP locus is one of the major causes of Rubinstein-Taybi syndrome with compromised CBP KAT activity and these patients are more prone to develop malignancy (Troke et al. 2006). Alteration of p300 gene has also been identified in various cancers of epithelial origin (Muraoka et al. 1996; Gayther et al. 2000). Mutation in p300 gene and loss of heterozygosity at p300 locus is reported to be associated with progression of colorectal, gastric, breast and brain cancer.

25.2.1.2 Virus Associated Diseases

p300 regulates integration of HIV in human genome by acetylating viral protein integrase (Cereseto et al. 2005). In HIV infected cells, LTR promoters are transcriptionally inactive due to chromatinization but are regulated by protein acetylation. HIV1 protein Tat relieves this inhibition by recruiting p300 and CBP in the viral LTR (Marzio et al. 1998). p300/CBP interacts with HIV Tat protein and acetylates Tat thereby activates the Tat dependent transcription of HIV genes on the integrated provirus (Deng et al. 2000; Ott et al. 1999). Interaction with Tat enhances the KAT activity of p300. KAT activity of p300 is essential to derepress the HIV-1 chromatin structure and thus activate the transcription of the integrated viral DNA *in vivo*. Apart from several cellular transcription factors, IRF family proteins especially IRF-1 can stimulate HIV1 LTR transcription in absence of Tat. p300/CBP and PCAF can acetylate IRF-1. IRF1 also recruits CBP in the promoter of HIV1 LTR in absence of Tat. Acetylation of the histone chaperone nucleophosmin (NPM1) by p300 is essential for the nuclear localisation of Tat and activates transcription of the integrated HIV provirus (Gadad et al. 2011).

25.2.1.3 Diabetes

Recently KATs and KDACs have been found to be associated with diabetes (Described in detail in Chap. 19 by Reddy and Natarajan of this collection). Diabetes

is associated with over expression of various vasoactive factors and extracellular matrix (ECM) proteins leading to alteration of morphology and function of organs which are also affected by complications arising due to chronic diabetes (Brownlee 2001). Glucose induces expression of fibronectin and other vasoactive factors in glucose induced endothelial cells through activation of NF kB and Activating protein 1 (AP1) (Chen et al. 2010). The mechanism of p300 expression by high glucose levels is not yet elucidated but the possible reasons could include activation of PKC and PKB, MAP kinase activation and oxidative stress induced PARP activation (Kaur et al. 2006; Xu et al. 2008; Chen et al. 2010). High glucose levels induce binding of p300 in the promoters of ET-1 and FN gene and cause acetylation of histones. Due to the acetylation of the promoters, various transcription factors are recruited which leads to the expression of vasoactive factors and ECM proteins. siRNA mediated silencing of p300 in human umbilical vein endothelial cells (HUVECs) effectively inhibit glucose mediated upregulation of vasoactive factors and ECM proteins.

25.2.1.4 Cardiac Hypertrophy

Agonist induced hypertrophy of cardiomyocytes is associated with enhanced transcriptional activity of p300 (Gusterson et al. 2002). p300 plays various essential roles in differentiation, growth and apoptosis of cardiac myocytes. During myocardial cell hypertrophy, GATA4 (a zinc finger protein) is upregulated. p300 acetylates GATA4 and enhances its DNA binding ability (Yanazume et al. 2003) which further induces the expression of ANF, ET-1, and α-MHC genes (Dai and Markham 2001). Thus, p300 mediated acetylation of GATA4 plays a crucial role in the development of myocyte hypertropy. Minute increase in the level of p300 is sufficient for the manifestation of myocardial hypertrophy by acetylating MEF2. p300 mediated acetylation and thus activation of MEF2 is also pivotal for driving the expression of hypertrophy associated genes (Wei et al. 2008).

25.2.1.5 Memory Impairment and Neurodegenerative Diseases

Information can be stored in the brain as short term memory which lasts for several minutes to hours. Short term memory can be stabilized to long-term memory which lasts for years. This stabilization of short term memory into long term memory requires new gene expression. CBP KAT activity is crucial for this stabilization of short term memory into long term memory. CBP is also required for transcriptional regulation for long term memory formation. Neurotrophic deprived primary cultured cerebellar neurons show decreased histone H3 and H4 acetylation with subsequent disappearance of CBP. Loss of function of CBP/p300 KAT activity during apoptosis and neurodegenerative diseases suggests its possible role in the disease progression (Rouaux et al. 2003).

25.2.2 Involvement of PCAF/GCN5 Family in Diseases

The PCAF/GCN5 family involvement has been shown indirectly in different diseases; we shall discuss their roles in cancer, HIV, malaria and diabetes.

25.2.2.1 Cancer

Twist1 is a basic helix-loop-helix transcription factor, is associated with tumor growth by the expression of Y-box binding protein 1(YB1) (Shiota et al. 2008). PCAF directly interacts and acetylates Twist1 and controls its intracellular localization and transcriptional activity. Knockdown of PCAF retards cell growth and invasive ability of urothelial cancer cells (Shiota et al. 2010). PCAF also plays important role in the tumor suppressor function of p53. PCAF protein levels are drastically reduced in intestinal type gastric cancer (ITGC). PCAF could efficiently suppress tumorigenicity of gastric cells and inhibit cells entering S phase from G1 phase (Ying et al. 2010).

25.2.2.2 HIV

One of the most essential events of HIV replication cycle is the integration of the viral DNA in the host genome. This function is performed by the viral protein integrase. Acetylation of integrase is one of the deciding factors for this activity. Apart from p300, GCN5 also acetylates HIV integrase protein and hence contributes to HIV infection (Terreni et al. 2010). Tat, a HIV encoded protein gets acetylated by both p300/CBP and PCAF (Kiernan et al. 1999) with functional consequence in the HIV life cycle.

25.2.2.3 Parasite Related Diseases

Plasmodium falciparum GCN5 (PfGCN5) has a conserved activity (Fan et al. 2004) and has preferences for acetylating histone H3K9 and K14 of plasmodium (Miao et al. 2006). Other KAT genes (MYST family member, PF11_0192) and two other KAT proteins (PFL1345 and PFD0795w) have also shown to be encoded by the plasmodium genome. Different acetylation marks are abundant throughout the erythroid cycle. PfGCN5 is actively recruited to various gene promoters to mediate histone acetylation and thus activate transcription of malarial proteins (Cui et al. 2007). Mono allelic expression of *var* gene is regulated by histone acetylation and thereby provides the antigenic switching and virulence of the parasite (Tonkin et al. 2009; Cui and Miao 2010; Petter et al. 2011). Thus acetylation of various malarial histones could be essential for the regulation of genome wide gene expression during erythroid development of the malaria parasite.

25.2.2.4 Diabetes

Type 2 diabetes mellitus (T2DM) is a disease caused by elevated glucose production characterized with insulin resistance, hyperinsulinaemia and hyperglycemia (Mitrakou et al. 1992; Perriello et al. 1997). In normal cells, plasma glucose levels are maintained within a very limited range by tightly maintaining the transcription regulation of phosphoenolpyruvate carboxykinase (PEPCK; gene code *Pck1*), the rate-limiting enzyme of hepatic gluconeogenesis. The peroxisome proliferator-activated receptor-g coactivator-1a (PGC1 α or PPARGC1A) is a crucial effector of pck1 (Yoon et al. 2001; Dentin et al. 2007). Upon activation, PGC1α forms complex with various transcription factors including FOXO1 and hepatic nuclear factor 4a (Hnf4a or Hnf4α), which subsequently induces the transcription of gluconeogenesis genes including pck1 (Yoon et al. 2001; Puigserver et al. 2003; Rhee et al. 2003). GCN5 (or KAT2A) acetylates PGC1α and deactivates which in turn causes suppression of gluconeogenic gene expression (Caton et al. 2010; Lerin et al. 2006).

25.2.3 Involvement of MYST Family KATs in Diseases

MYST is derived from three classical members of KAT family: mammalian MOZ, yeast Ybf2/Sas3 and Sas2, and mammalian Tip60. MORF and HBO1 discovered later are the other members of the MYST family. This family of acetyltransferases have been implicated in the DNA repair pathway and hence have intrinsic association with diseases such as cancer.

25.2.3.1 Cancer

Tip60 is an essential component for the chromatin repair mechanism. Tip60 mutations are associated with defects in DNA double strand break repair and provide resistance for the DNA damaged cells towards apoptosis (Ikura et al. 2000), which is a characteristic feature associated with cancers. Androgen receptor (AR) is a hormone dependent transcription factor which has a strong link with development of prostate cancer. Tip60 acetylates AR and activates its function (Brady et al. 1999; Gaughan et al. 2001, 2002). Tip60 has also been linked with tumor suppressor p14ARF. Upon genotoxic stress, p14ARF directly interacts with Tip60 resulting in activation of the ATM/CHK signaling cascade and cell cycle arrest at the G2 phase (Eymin et al. 2006). Tip60 acetylates retinoblastoma (Rb) tumor suppressor. Reports suggest that in colon and lung carcinoma tissues show significant reduced expression of Tip60 (LLeonart et al. 2006). Tip60 is also reported to acetylate K120 residue of tumor suppressor p53 and is associated with activation of p53 mediated cell cycle arrest and apoptosis. Acetylation of p53K120 by Tip60 is crucial for p53 mediated activation of pro-apoptotic genes BAX and PUMA. K120 acetylation does not confer stability to p53 nor enhances DNA binding, but it occurs upon DNA damage or oncogenic

stress (Tang et al. 2006). P53 K120 is also frequently mutated in various human cancers. Upregulation of Tip60 is also associated with onset of epithelial tumorigenesis (Hobbs et al. 2006).

MOZ (monocytic leukaemia zinc-finger protein) forms a fusion protein with CBP by chromosomal translocation in AML (Borrow et al. 1996). MOZ also forms in-frame fusions with CBP and its homologue p300 (Kitabayashi et al. 2001); (Panagopoulos et al. 2002; Rozman et al. 2004) nuclear receptor coactivator TIF2 (Carapeti et al. 1998; Kindle et al. 2005) and NcoA3. The coding region of MOZ is identified as a common retroviral insertion site which could be its reason for translocation in leukemia (Lund et al. 2002). The catalytic domains of these fusion proteins remain functionally active and due to misdirection of the targeting domains the various histone and nonhistone proteins get aberrantly acetylated. Apart from AML, in benign uterine smooth muscle tumours, MORF (MOZ related factor) coding region gets disrupted by another chromosomal translocation (Moore et al. 2004). Loss of MORF and the manifestation of the tumor could be due to decrease in the stability of the ING5-MOZ/MORF-BPRF complex (Doyon et al. 2006) and subsequently affect the ability of ING5 to modulate p53 activity (Shiseki et al. 2003). MOZ is overexpressed in chemical hepatocarcinogenesis but chromosomal translocation has not been identified. Human MOF acetylates histone H4K16 in cells (Smith et al. 2005) and this modification is a critical regulator of various chromatin related processes and is also linked to several diseases (Shia et al. 2006). Most importantly, loss of H4K16 acetylation is reported to be one of the frequent hallmarks of various cancers (Fraga et al. 2005). H4K16 acetylation inhibits chromatin higher order structure (30 nm fiber) formation and also interferes with various chromatin remodelling enzymes (Shogren-Knaak et al. 2006). Loss of H4K16 acetylation by downregulation of HBO1 results in reduced cell cycle arrest (Smith et al. 2005).

25.2.3.2 HIV

HIV1 Tat protein suppresses the transcription of Mn-SOD gene by inhibiting Tip60 KAT activity (Creaven et al. 1999). Downregulation of Mn-SOD gene is one of the factors for the generation of oxidative stress leading to T-cell depletion in AIDS (Westendorp et al. 1995). Tat has also been reported to be responsible for degradation of Tip60 by ubiquitination (Col et al. 2005).

25.2.3.3 Malaria

MYST acetyltransferase has been identified in *Plasmodium falciparum* (malarial parasite) genome. PfMYST preferentially acetylates histone H4 at K5, K8, K12 and K16 in vitro. PfMYST localizes to both nucleus and cytoplasm. These functions are essential for the transcriptional regulation, cell cycle progression and DNA damage repair of the parasite. pfMYST is also reported to be recruited to the *var* gene promoter and acetylates H4 to facilitate transcription initiation (Miao et al. 2010).

The significant contribution of the acetyltransferases towards disease manifestation and progression, has led to increased speculation about considering the modulators of these enzymes as potential therapeutic agents. However, the specific inhibitor or activator for a particular enzyme could prove to be an extremely valuable therapeutic strategy. In this section we will discuss briefly about various KAT inhibitors and activators reported and their mode of action. Lastly we will give a brief overview about the possible role of these modulators in disease therapeutics as well as the potential combinatorial therapy.

25.3 Modulators of Histone Acetylation

25.3.1 Inhibitors of Histone Acetylation

The concept that reversible histone acetylation could be a therapeutic strategy was first established by KDAC inhibitors. Some KDAC inhibitors are already in clinical trials for their potential role in cancer treatment. Suberoylanilide hydroxamic acid (SAHA, vorinostat) have been now approved by U.S. Food and Drug Administration (FDA) for treatment against advanced cutaneous T-cell lymphoma (Mann et al. 2007; Richon et al. 1998). In comparison to KDAC inhibitors, use of KAT modulators against diseases is a relatively new field. Extensive research has been employed to identify various KAT modulators and the strategies followed are: rational design, high-throughput screening of synthetic libraries, and enzymatic screens with natural products (Cole 2008). The inhibitors of acetyltransferases are comparatively more in number with respect to the acetyltransferase activators.

Among different KATi, inhibitors of p300/CBP and PCAF/GCN5 have been more extensively worked on. Among all the KAT inhibitors, the first reported KAT inhibitors are the bisubstrate analogue Lys-CoA and H3-CoA-20 (Lau et al. 2000). These inhibitors were designed on the basis of the knowledge that upon substrate binding the target enzyme employs a ternary complex, involving the H3 peptide and acetyl CoA. Lys-CoA one of the simple analogue of peptide CoA is a potent and specific inhibitor for p300. H3 CoA is a 20 amino acid peptide derived from histone H3 tail and conjugated with Coenzyme A and specifically inhibits PCAF. Bisubstrate analogues were also used to develop inhibitors against Tip60 and Esa1 (yeast orthologue of Tip60). Among others, H3K16 CoA proved to be the most potent inhibitor of Tip60 and Esa1 (Wu et al. 2009). But these bisubstrate inhibitors were not cell permeable and hence their use is restricted.

The first reported natural KAT inhibitor is anacardic acid (nonadecyl salicylic acid) isolated from cashewnut shell liquid. Anacardic acid non specifically inhibits KATs p300, PCAF and Tip60. Due to its non specificity against any particular enzyme and lack of cell permeability, the scaffold has been exploited for the synthesis of better inhibitors. A salicylate derivative of anacardic acid has been developed with specificity towards PCAF, which is reported to have twofold improved potency

to inhibit recombinant PCAF enzyme and has also been shown to inhibit PCAF mediated acetylation in HEP G2 cell lines (Ghizzoni et al. 2010). Curcumin, a polyphenolic compound isolated from *Curcuma longa* rhizome is a specific inhibitor of p300. Curcumin possesses anticancer effects by inducing apoptosis in cancerous cells but not in healthy normal cells, whereas one of the major disadvantage of curcumin is that it has a very poor bioavailability. As an improvement, our group has synthesized and characterized CTK7A, a water soluble derivative of curcumin which inhibits p300/CBP and PCAF mediated acetylation and has an anti oral cancer effect (Arif et al. 2010). However, the bioavailability of CTK7A in comparison to curcumin is yet to be investigated.

Another naturally isolated inhibitor of p300 and PCAF is garcinol. Garcinol is polyisoprenylated benzophenone isolated from the edible fruit, *Garcinia indica* or kokum fruit (Balasubramanyam et al. 2004). The IC_{50} values of garcinol for p300 and PCAF is 7 and 5 μM respectively. Intramolecular cyclisation of garcinol yielded isogarcinol. Further substitution of the 14th position yielded 14-isopropoxy IG (LTK-13) and 14-methoxy IG (LTK-14) and disulfoxy IG (LTK-19). LTK13, 14 and 19 could selectively inhibit p300 but not PCAF (Mantelingu et al. 2007b). The mechanistic details of the specificity of LTK14 against isogarcinol and garcinol have also been reported. LTK14 acts as a noncompetitive inhibitor for acetyl CoA and histones unlike non specific inhibitors isogarcinol and garcinol. LTK14 has a single binding site on the p300 KAT domain whereas both garcinol and isogarcinol has two binding sites (Arif et al. 2009). The specificity of LTK14 towards p300 is provided by the formation of the lactone ring upon cyclization of garcinol. The methyl group present on the aromatic phenol of LTK14 reduces its affinity towards PCAF and not p300. Further studies revealed that LTK14 alters the tertiary structure of p300 whereas garcinol and isogarcinol alters the secondary structure of p300. Recently, plumbagin has been reported to be a p300 specific KAT inhibitor (Ravindra et al. 2009). It is a hydroxynaphthoquinone and is isolated from the roots of the medicinal plant, *Plumbago rosea*. Plumbagin follows the non competitive mode of inhibition for p300 and its single hydroxyl group has been shown to be crucial for its KAT inhibitory activity. As most of the KAT inhibitors possess polyhydroxyl groups, it is speculated that this chemical moiety might be a decisive factor for acetyltransferase inhibition.

Derivatization of γ-butyrolactones yielded MB-3 which is an inhibitor of CBP and Gcn5 with an IC_{50} of 100 μM. The length of the aliphatic side chain of MB-3 is critical for its KAT inhibitory activity (Biel et al. 2004). Isothiozalones have also proved to be potent inhibitors of p300 and PCAF. But these compounds are highly reactive and have off targets as they have high chemical reactivity with free thiol groups of other proteins. Recently, epigallocatechin-3-gallate (EGCG), a component present in green tea has been shown to posses KAT inhibitory potential. It showed inhibitory effects towards majority of the KATs but not other chromatin modifying enzymes (Choi et al. 2009). C646, a pyrazolone-containing small molecule obtained after in silico based virtual ligand screening specifically inhibits p300/CBP KAT and also shows anticancer activities (Bowers et al. 2010).

25.3.2 Small Molecule Activators of Acetyltransferases

KAT activators are small molecules that can activate the acetyltransferase activity of a KAT by possibly altering the structural configuration of the enzyme. The first reported and best characterized KAT activator is N-(4-Chloro-3-trifluoromethyl-phenyl)-2-ethoxy-6-pentadecyl-benzamide (CTPB) (Balasubramanyam et al. 2003). CTPB can efficiently induce histone acetylation levels both *in vitro* and *in vivo* (Selvi et al. 2008). Upon binding to p300, CTPB induces structural alteration of p300 and further leads to autoacetylation. Detailed structural and functional studies led to the conclusion that the $–CF_3$ and –Cl at the para position of CTPB is crucial for its KAT activation property (Mantelingu et al. 2007a). Due to its impermeability to cells, CTPB has been conjugated on glucose derived carbon nanosphere (CSP) by simple adsorption to achieve in vivo activation. Another recently reported KAT activator is nemorosone, which belongs to a group of polyhydroxybenzophenones. Like CTPB, Nemorosome also activates p300 KAT activity and the possible mechanism of activation is also by binding to the same region of p300 where CTPB binds (Dal Piaz et al. 2010). Presently, isolation and characterization of more specific activators of KATs are in progress for use as possible future generation therapeutics.

25.4 KAT Modulators as Possible Future Generation Therapeutics

Histone hyperacetylation has been causally associated with several diseases such as inflammatory disorders, viral diseases etc. Hence, the use of histone acetylation inhibitors should in theory be capable of atleast partially reversing these effects. Most of these studies have been done on cell culture based assays. However, the results obtained have provided enough evidence to support the possibility of considering the KAT modulators as a therapeutic strategy.

25.4.1 Histone Acetylation Inhibitors as a New Generation Therapeutic Strategy

Anacardic acid can induce apoptosis in a TNFα or chemotherapeutic agent dependent manner and could efficiently downregulate transcription of genes essential for invasion, proliferation, survival and angiogenesis. Anacardic acid could inhibit constitutive expression of NFκB and also inhibit acetylation and nuclear translocation of p65. Anacardic acid also inhibits PfGCN5 activity and thereby downregulates developmentally regulated genes of *Plasmodium falciparum* (Cui et al. 2008).

Being a specific inhibitor for p300/CBP, curcumin inhibits HIV virus proliferation and acetylation of HIV Tat. Apart from HIV, curcumin has also proved to be a potential anticancer, antiviral, antiarthritic, anti-amyloid, antioxidant, and anti-inflammatory agent (Zhou et al. 2011). CTK7A (Sodium 4-(3,5-bis(4-hydroxy-3-methoxystyryl)-1H-pyrazol-1-yl)benzoate), the water soluble derivative of curcumin induces histone hypoacetylation in oral cancer cell lines and promote senescence and growth arrest. Treatment of CTK7A also reduces tumor size in oral cancer xenografted nude mice (Arif et al. 2010). The polyisoprenylated benzophenone garcinol induces apoptosis in HeLa cells and induces global gene repression (Balasubramanyam et al. 2004). LTK14, a derivative of garcinol inhibits HIV multiplication, inhibits viral syncia formation in HIV infected cells and is nontoxic to T cells. Plumbagin has also proved its therapeutic activity by inhibiting NFκB pathway. Epigallocatechin-3-gallate (EGCG) isolated from green tea has antiproliferative properties against colon, lung, and breast cancers and chronic inflammation especially by its anti-NF-κB transactivation activity and subsequently inhibiting EBV induced B-cell transformation (Choi et al. 2009). C646 has also strong anticancer activities due to its p300 specific inhibition (Bowers et al. 2010). By inhibiting p300 acetyltransferase activity, it decreases AR function and downregulates p65 which further induces apoptosis in prostate cancer cells (Santer et al. 2011). Isothiazolones derivatives have possible implications in colon cancer treatment (Stimson et al. 2005).

25.4.2 Therapeutic Perspective of KAT Activators

Histone acetylation is altered in various diseases and the balance between acetylation and deacetylation is hampered. Various diseases including cancers and neurodegenerative disorders are associated with reduced expression of p300 and CBP. KDAC inhibitors have been extensively used to reactivate acetylation and a few have already entered in clinical trials. KDACi treatment has also provided promising results in treatment of glioblastoma. The major limitation of using KDAC inhibitors is the lack of specificity for any particular KDACs. KDACs functions in association with the other members of the family and the loss of one protein generally gets compensated by other. Thus KDAC inhibitors induce global histone acetylation. Various diseases are manifested with alteration of KAT activity of a particular acetyltransferases and thus KAT activators specific for a particular enzyme could induce its activity and as a consequence reactivate its function. KAT activators also induce histone hyperacetylation, but unlike KDACi it specifically activates its target KAT. CTPB specifically activates acetyltransferase activity of p300 and CBP. p300 and CBP activity is reported to be lost in various neurodegenerative disorders and thus CTPB could be a potential therapeutic agent in the treatment of the disease. The lack of cellular permeability limits CTPB to be used in animal model. Also the major hurdle for drugs used for brain targeting for diseases such as glioma is to cross the blood brain barrier (BBB).

25.4.2.1 Nanomaterial as Delivery Vehicle for Activators

Often the failure to access the drugs across the blood brain barrier in therapeutically required quantities is one of the major impediments in looking out for alternative drug molecules to treat many central nervous system related diseases like Alzheimer's, Huntington's, Parkinson's etc. (Jain 2011). The blood brain barrier which segregates the blood compartment of the brain from the extracellular fluid, is composed of a monolayer of endothelical cells connected through tight intercellular junctions. This barrier restricts the diffusion of many hydrophobic drugs (also active peptides and proteins) into the brain (Kreuter et al. 1995); (Brasnjevic et al. 2009). These limitations can be circumvented by the use of nanoparticles whose size range vary from 1 nm to few hundred nm and are perfectly suitable to interact with biological cells, proteins and DNA (Malam et al. 2011). Furthermore, the size dependent optical, magnetic and chemical properties of many of the nanoparticles at the quantum regime facilitate their use in diagnostics, imaging and drug delivery. Some of the critical issues limiting the pharmaceutical design, like the delivery of poorly soluble drugs, targeting the drug at particular site, blood brain barrier, and simultaneous delivery of two or more drugs can be effectively addressed by using nanoparticles. Targeted delivery of drugs using nanoparticles not only alter the pharmacokinetic profile of the drugs but also reduce their systemic toxicity by preventing their accumulation in other parts of the body. Polymeric nanoparticles, solid lipid nanoparticles, cyclodextrin nanoparticles, liposomes, and inorganic nanoparticles such as mesoporous silica, gold, CdSe, Fe_3O_4, fullerene, carbon nanotubes are widely used for diagnostic, imaging and drug delivery applications (Emerich and Thanos 2006). Delivery of drugs in different cell lines using these nanoparticles have been demonstrated, both in *in vitro* and *in vivo* experiments. However, when it comes to brain cells, most of these nanoparticles fail to deliver and demands special modifications as they have to cross the BBB. Receptor mediated endocytosis pathway in which, the strong affinity between a macromolecule (linked to the nanoparticles) and its specific receptor (expressed on the cell surface) is normally exploited for the delivery of the drug into the brain cells through nanoparticles. Large neuropeptides such as insulin, transferrin, and leptin cross the BBB through the receptor mediated endocytosis mediated by the corresponding receptors (Gupta et al. 2005); (Duffy and Pardridge 1987). Decorating such molecules on the surface of the nanoparticles would be expected to help them cross the BBB. For example, PLGA, the poly(lactic-co-glycolic acid) nanoparticles were shown to cross the BBB in rat brain only when it was modified with peptides (Costantino et al. 2005). Similarly, transferrin coated, drug loaded PLGA nanoparticles are shown to deliver the drug across the BBB through receptor mediated endocytosis mechanism as the BBB expresses transferrin receptor excessively (Chang et al. 2009). In an another approach, surfactant (polysorbate 80) coated PLGA nanoparticles(loaded with doxorubicin drug) were shown to enhance the adsorption of apolipoproteins which in turn, facilitated the receptor mediated endocytosis of the PLGA nanoparticles (Gelperina et al. 2010). Solid lipid nanoparticles which are coated with PEG (polyethylene glycol)

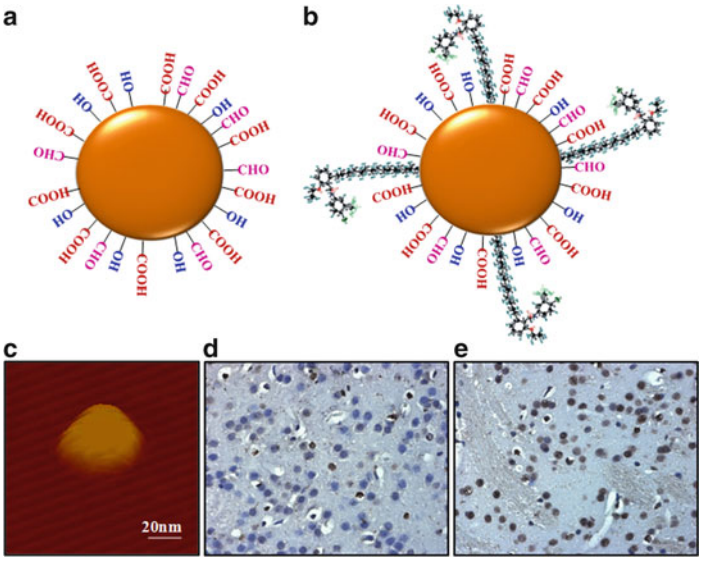

Fig. 25.2 Carbon nanosphere as a vehicle to carry KAT activators to mice brain. (a) Pictorial representation of Carbon nanosphere (CSP) showing the charged residues on the surface, **(b)** CTPB adsorbed on the surface of carbon nanosphere. **(c)** AFM image of chemically conjugated CSP CTPB. CSP CTPB was coated on the surface of freshly cleaved mica plate and was visualized under AFM (Chatterjee et al. 2010) **(d)** Immunohistochemistry images of mice treated with **(d)** CSP and **(e)** CSP CTPB in mice brain using anti-acetylated histone H3 antibody

to avoid the reticuloendothelial system clearance and thiamine ligands which binds with the thiamine receptors are transported to the brain through the receptor mediated endocytosis (Kaur et al. 2008). Recently, carbon nanospheres are shown to cross the blood brain barrier (Selvi et al. 2008). Without any surface modification probably due to the presence of oligosaccharide side chains containing terminal glucose, mannose residues mimicking the receptor mediated endocytosis internalization of such macromolecules.

25.4.2.2 Histone Acetylation Activation Across the BBB and Its Implications

Glucose derived carbon nanosphere CTPB conjugate, easily cross the BBB and induces histone acetylation in the brain (Fig. 25.2). Thus KAT activator conjugated with CSP could specifically activate acetyltransferases activity in brain and be used as a potential therapeutic agent. This delivery system of KAT activators coupled with nanospheres could be extremely useful in the treatment of various neurodegenerative diseases. In Alzheimer's' disease, reduction in levels of histone acetylation (especially H4) in hippocampus is reported (Francis et al. 2009). Thus small molecule

activators, in combination with a targeted delivery system (like CSP) could be a potential therapeutic in treatments for Alzheimer's disease or other neurodegenerative diseases.

25.5 Future Perspectives

The modulators of histone acetylation have been tested for their effect on these disease conditions mostly in an in vitro condition. Before embarking on testing the therapeutic potential of these histone acetylation modulators, a detailed study with respect to their pharmacokinetics, bioavailability, the effect on gene expression, miRNA expression etc. needs to be understood. Since, these evidences will help in understanding their specific versus nonspecific effects in the physiology. However, the broader implications of the concept of acetylation being considered as a therapeutic target can also encompass the following aspects.

25.5.1 Combinatorial Therapy

Acetylation forms an important nodal point for several epigenetic crosstalks that regulate the process of transcription and hence, disturbing this centric modification may not necessarily influence the whole pathway. Especially in the case of pathophysiological conditions, several regulatory events are dysfunctional and hence to achieve a therapeutic effect it may be necessary to target not just the acetylation mark but also the other associated modifications such as lysine and arginine methylation marks with their specific modulators.

25.5.2 Targeted Delivery

The acetylation event is a global phenomenon essential for the process of gene expression and hence the modulation may lead to undesirable effects at other parts of the genome. Also, the small molecules have potential pleiotropic effects. Therefore, targeted delivery of these modulators with the help of nanomaterials might prove to be a more efficient strategy.

25.5.3 Novel Therapeutic Concepts

The cancer stem cell (CSC) populations are essentially in a non-replicating state, thus several of the frontline anti cancer drugs, fail to have any effect on these. The targeted acetylation modulators can be used to facilitate the differentiation of this

CSC population and then killed. On the other hand, several regenerative medicinal strategies are exploring the possibility of stem cell based therapies, the major limitation being the availability of these cells. With the advent of the iPS technology, the histone acetylation modulators can also be tested for the dedifferentiation process.

25.5.4 Pathway Alterations

There have been recent evidences implicating the role of histone acetylation in metabolic processes with important enzymes being identified as potential substrates. And it is also becoming evident that there indeed exists an acetylation centric epigenetic language that acts as a signalling event facilitating the process of transcription and gene expression. Hence, the use of modulators as a therapeutic strategy should be done only along with the information of the effect of these modulators on metabolism and other pathways.

Collectively, the modulators of acetylation are important candidates to be considered for their potential therapeutic applications based on the several preclinical investigations highlighted in this chapter. The efficacy and the effectiveness of such an effort is strengthened by the fact that deacetylase inhibitors are presently being used in clinical trials.

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Chapter 26
DNA Methylation and Cancer

Gopinathan Gokul and Sanjeev Khosla

Abstract Cancer has been considered a genetic disease with a wide array of well-characterized gene mutations and chromosomal abnormalities. Of late, aberrant epigenetic modifications have been elucidated in cancer, and together with genetic alterations, they have been helpful in understanding the complex traits observed in neoplasia. "Cancer Epigenetics" therefore has contributed substantially towards understanding the complexity and diversity of various cancers. However, the positioning of epigenetic events during cancer progression is still not clear, though there are some reports implicating aberrant epigenetic modifications in very early stages of cancer. Amongst the most studied aberrant epigenetic modifications are the DNA methylation differences at the promoter regions of genes affecting their expression. Hypomethylation mediated increased expression of oncogenes and hypermethylation mediated silencing of tumor suppressor genes are well known examples. This chapter also explores the correlation of DNA methylation and demethylation enzymes with cancer.

26.1 Introduction

Cancer is a complex and heterogeneous disease characterized by uncontrolled growth and cellular machinery that has gone haywire. In order to account for the diverse molecular changes that occur in different cancer types it has been conceptualized that cancer encompasses many diseases. Pathologists view cancer as acquiring properties of cells belonging to different developmental stages but appearing inappropriately in the tumors (Pitot 1986). The two-hit hypothesis proposed by Knudson (1971) remains the basis of correlating the genetic events with cancer

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initiation even today. For instance, in retinoblastoma, two genetic events are required in the retinal cell, which would result in the inactivation of both copies of the tumor suppressor – retinoblastoma gene (*RB*). In the familial set up where all cells of a person inherit a mutated allele of *RB*, a single mutation in the normal functional allele can give rise to cancer whereas in non-hereditary cancer, both mutations need to take place in the same cell. This genetic model of cancer has been supported by characterization of a wide array of molecular changes that occur among cancer types including the mutations which in small numbers are seen in benign, non-invasive tumors and the large-scale genetic changes and genetic instability found in invasive and metastatic tumors (Feinberg et al. 2006). However, there are a few shortcomings to the Knudson's two-hit model. Firstly, the model does not take into account the heterogeneity and complexity observed in tumors and secondly, tumors that show aneuploidy, polyploidy, and complex karyotypes are often incompatible with the two-hit hypothesis (Dutrillaux et al. 1991). Moreover, and of late it is becoming amply clear that genetic mechanisms alone cannot account for all the diverse molecular changes seen in tumors. Epigenetic mechanisms which provide an additional layer of control for gene expression have been proposed to be an important factor in cancer development and postulated to provide for one of the hits required for carcinogenesis.

26.2 Cancer as an Epigenetic Disease

The term "epigenetics" defines the study of all meiotically and mitotically heritable changes in gene expression patterns that are not coded in the DNA sequence itself (Egger et al. 2004). Epigenetic modifications like DNA methylation and histone modifications can bring about changes in gene function or dosage similar to those obtained in case of mutations, chromosomal rearrangements and gene duplications. Studies in tumor tissues have revealed that a given gene can exhibit multiple epigenetic changes just as the numerous possible genetic alterations (Feinberg et al. 2006). Thus, integrating the data on epigenetic regulation of gene expression with their genetic alterations is of utmost importance for understanding the mechanisms underlying cancer development. In 1983, through the pioneering efforts of Andrew Feinberg and Bert Vogelstein, loss of methylation at CpG dinucleotides in cancer cells was identified, and can be considered as the first report on the correlation of epigenetics with cancer. They found that a substantial proportion of CpG dinucleotides in the human genome had lost DNA methylation in cancer cells (Feinberg and Vogelstein 1983). Around the same time, Ehrlich's group also found that the global levels of 5-methylcytosine were reduced in cancer cells (Gama-Sosa et al. 1983). Though there was initial skepticism about this correlation, today the role of DNA methylation has become well established in multiple cancers (Jones and Baylin 2002; Feinberg and Tycko 2004). This loss of methylation has been reported in every tumor type studied; both benign and malignant; and interestingly even pre-malignant adenomas exhibit altered DNA methylation (Goelz et al. 1985; Feinberg et al. 1988).

Although hypomethylation of CpG islands was the first epigenetic change identified in cancers, it has not been given as much importance as has been to hypermethylation of CpG islands. Baylin and colleagues in 1986 identified site-specific hypermethylation of *CALCITONIN* gene in small-cell lung cancer (Baylin et al. 1986). The first correlation of CpG island hypermethylation with the inactivation of a tumor-suppressor gene was established for the Retinoblastoma gene (*RB*) when *RB* promoter was found to be methylated in a significant subset of sporadic and hereditary retinoblastomas (Greger et al. 1989). Promoter hypermethylation has now been shown for several other loci to be the cause of gene inactivation in cancer. Hypermethylation is observed within the CpG islands of specific genes and represents a change in 5-methylcytosine distribution at specific loci in the genome rather than an overall increase in the total amount of methylation. In many cases, this *de novo* methylation of CpG islands occurs early in the process of carcinogenesis and surprisingly can even be detected in the apparently normal epithelium of patients-a process that is associated with aging (Issa et al. 1994; Ahuja et al. 1998) and inflammation (Coussens and Werb 2002; Nelson et al. 2004; Lu et al. 2006). In fact, aging is considered the most important risk factor for the development of most malignancies of adulthood as DNA methylation patterns in aging cells have a tendency to be aberrant, similar to what is seen in transformed cells (Neumeister et al. 2002).

Another important link that has broadened the role of epigenetics in cancer is the correlation between chromatin organization and DNA methylation. The earliest of experiments probing this link were done in the Cedar and Graessmann laboratories. They found that pre-methylated, naked DNA templates when transfected or microinjected into cells became transcriptionally silent after getting packaged into a repressive form of chromatin (Keshet et al. 1986; Buschhausen et al. 1987). Foremost amongst the chromatin modifications are the covalent modifications of histones that can change the chromatin conformation and control gene activity. Cytosine methylation can attract methylated DNA-binding proteins and histone deacetylases to the methylated CpG island, leading to chromatin compaction and gene silencing (Jones et al. 1998; Nan et al. 1998). This has further strengthened the link between the two major epigenetic components of gene regulation. In addition, correlation has also been made between covalent histone modifications and nucleosomal remodeling (Esteller 2006). It is now well established that the three processes of DNA cytosine methylation, histone modification and nucleosomal remodeling are intimately linked and that alterations in these processes result in the epigenetic changes which would lead to events like the permanent silencing of cancer-relevant genes and genomic instability. Working on mouse models, Jaenisch and colleagues demonstrated that *Dnmt1* hypomorphic mutation reduces the frequency of intestinal neoplasia when crossed to Apc^{Min} mice (Gaudet et al. 2003) whereas, a high frequency of lymphomas were observed in mice with hypomorphic *Dnmt1* allele (Eads et al. 2002). Together, these data indicated that cancer risk is associated with a disruption in the balance of methylation rather than hypomethylation and hypermethylation *per se*.

Based on the subtle differences observed in the epigenetic profiles of stem cells and the cancer cells, several researchers have proposed the theory of cancer stem

cells, which suggests that stem cells are the more likely targets of epigenetic disruption leading to cancer. “Cancer stem cells” (CSCs), which constitute a small minority of neoplastic cells within a tumor, are the abnormal stem cells generated from normal stem cells upon accumulation of series of progressive genetic/epigenetic changes. CSCs are now well characterized in the hematopoietic system and colon cancers (O’Brien et al. 2007; Mani et al. 2008). The incompetence of many of the anti-cancer chemotherapeutic agents has been attributed to the presence of cancer stem cells, against which these drugs are less effective (Ren 2005).

Thus, in the past 20 years, cancer epigenetics has transformed into a full-fledged field with a focus on the different mechanisms involved in epigenetic regulation including DNA methylation, histone modifications and nucleosomal remodeling. In addition, the discovery of epigenetically regulated imprinted genes and their role in cancer has also added another dimension to this field (Jirtle 1999; Jelinic and Shaw 2007; Hirasawa and Feil 2010). While the finer mechanistic detail of the role of epigenetic modifications in cancer progression still needs to be worked out, epigenetic events are recognized as possibly one of the hits required in Knudson’s hypothesis (Yu and Shen 2002). Initially, the clonal genetic model of cancer was widely accepted, wherein cancer arises through a series of mutations in oncogenes and tumor suppressor genes. This would give rise to a monoclonal population of tumor cells and epigenetic changes were viewed as surrogate correlates of cancer. But according to the recently proposed epigenetic progenitor model, cancer begins with an epigenetic alteration of stem/progenitor cells within a tissue, which is followed by a gatekeeper mutation involving a tumor suppressor gene or an oncogene, leading to genetic and epigenetic instability (Feinberg et al. 2006).

26.3 DNA Methylation and Cancer

The epigenetic landscape is maintained by interplay between the key modifications: DNA methylation, histone tail modifications, and small RNA molecules. However, in this review we focus on the interplay between DNA methylation machinery and carcinogenesis. In the altered epigenetic setup of a cancerous cell, aberrant DNA methylation is one of the most well studied epigenetic mechanisms affecting gene expression. Collated below is data pertaining to the role of DNA methylation machinery in establishing the normal and altered epigenetic makeup of the human genome.

26.3.1 CpG Islands and CpG Methylation

Methylation of DNA is a stable modification and can be inherited through cell divisions. Methylation of DNA in mammals primarily takes place at the 5′ position of cytosine in the context of 5′-CG-3′ dinucleotide (Sinsheimer 1955; Bird 2002).

Although non-CpG methylation has been reported in mammals (Ramsahoye et al. 2000), its presence is miniscule as compared to CpG methylation. The haploid human genome contains approximately 50 million CpG dinucleotides, which represents $2^{50,000,000}$ different permutations of CpG methylation. CpG dinucleotides are unevenly distributed across the human genome with vast DNA stretches deficient in CpGs, interspersed by CpG clusters called CpG islands (Laird 2003). CpG islands were identified almost 20 years ago on the basis of the strikingly discordant patterns of digestion of genomic DNA by restriction enzyme isoschizomers that differed only by their sensitivity to cytosine methylation (Singer et al. 1979). CpG islands have been defined by different groups based on various criteria. Initially they were defined as stretches of DNA (200 bp or longer) with a (G+C) content of 0.50 or greater and an observed to expected CpG dinucleotides ratio of 0.60 or greater. Later on, Takai and Jones (2003) found that increasing the size threshold to 500 bp and the (G+C) content threshold to 0.55 biased the definition against repetitive sequences and included only unique CpG sequences. According to computational estimates, there are at least 29,000 CpG islands in the human genome (Lander et al. 2001; Venter et al. 2001). CpGs are vastly under-represented in the genome mainly because deamination of cytosine gives rise to uracil, which is recognized as foreign in the DNA and hence replaced. Moreover, deamination of methylcytosine gives rise to thymine, which is less readily recognized as foreign and therefore prone to mutation (C:G to T:A transition) and depleted in the genome (Duncan and Miller 1980). Interestingly, CpG islands are found at the promoter sites of approximately 50% of the genes in the human genome (Loshikhes and Zhang 2000), most of which are the 'housekeeping' genes, where they are kept free from methylation (Larsen et al. 1992; Ponger et al. 2001). On the other hand, CpG islands within transposable elements are heavily methylated (Yoder et al. 1997). The methyl group in CpG dinucleotides protrudes from the cytosine nucleotide into the major groove of the DNA and has two main effects: it displaces transcription factors that normally bind to the DNA; and it attracts methyl binding proteins, which in turn are associated with gene silencing and chromatin compaction (Fazzari and Greally 2004). Methylation of DNA in the common 'B' form facilitates a conformational change to the 'Z' form, increases the helical pitch of DNA and alters the kinetics of cruciform extrusion (Murchie and Lilley 1989; Zacharias et al. 1990). Promoters with a few exceptions are generally inactive when the CpG island within them are methylated (an exception, for example, is seen in the case of the H-2K gene promoter, Tanaka et al. 1983). The transcriptional machinery can respond to CpG methylation in different ways. Either methylation at specific sites within the CpG island can interfere with the efficiency of expression (as seen in the case of Herpes simplex thymidine kinase and the Epstein-Barr virus latency C promoters) or it can be the density of methylation (human α- and γ-*GLOBIN*, mouse *MyoD1* promoter) that is responsible for interfering with transcription (Zingg and Jones 1997). Moreover, since the structure of 5-methylcytosine is similar to thymine, the methylation of cytosine might lead to generation of new consensus sequences for some transcription factors. This has been seen in the case of methylation at CpG in a low affinity AP-1 binding site that converts it to high affinity site (Tulchinsky et al. 1996) or in the CRE sequence

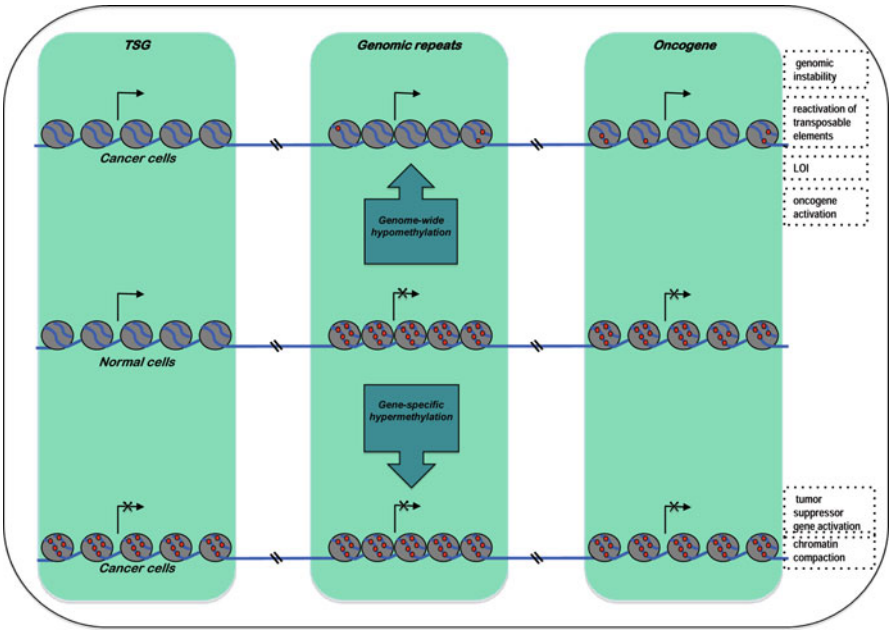

Fig. 26.1 Aberrant methylation during cancer progression. In normal cells, while promoter CpG islands of most genes lack methylation, repeat elements and promoters of oncogenes are kept inactive by DNA methylation. Genome-wide hypomethylation causes activation of oncogenes and activates aberrant transcripts from within repeat elements leading to genomic instability. On the other hand, hypermethylation of tumor suppressor genes (TSG) leads to their silencing. *LOI* loss of imprinting, *TSG* tumor suppressor gene. *Raised arrows* denote transcriptional activation; *crossed arrows* denote transcriptional repression. *Red circles*: Methyl CpG and *circles* with *curved lines* denote nucleosomal organization of chromatin

which enhances the binding of C/EBPα transcription factor (Rishi et al. 2010). Similarly, the binding of several other DNA binding proteins (AP-2, Ah receptor, CREB/ATF, E2F, EBP-80, c-Myc, NF-κB) is inhibited when the CpG within their recognition sequence is methylated (Jones and Gonzalgo 1997).

26.3.2 Aberrant DNA Methylation in Cancer

Global hypomethylation and gene-specific hypermethylation are the hallmarks of most cancers studied (Fig. 26.1 and Feinberg and Tycko 2004). Global alterations in DNA methylation have been observed not only in fully developed cancers, but also in the precancerous stage, including chronic inflammation, persistent viral infection and cigarette smoking. Furthermore, aberrant DNA methylation is significantly associated with aggressiveness of cancers and the poor outcome in cancer patients.

26.3.2.1 Genome-Wide Hypomethylation

The earliest evidence linking DNA methylation to cancer came with the discovery of global hypomethylation in tumors (Riggs and Jones 1983). Many CpG islands are normally methylated in somatic tissues (Strichman-Almashanu et al. 2002). During global hypomethylation, these methylated islands can become hypomethylated in cancers activating the nearby genes. High-throughput genomic DNA methylation studies have identified that the frequency of hypomethylated sites might be quite high in tumors and numerous genes have been identified that lose DNA methylation in different cancers (Adorjan et al. 2002; Lacobuzio-Donahue et al. 2003). The important link between DNA hypomethylation in cancer and chromosomal instability was established by Ehrlich and colleagues (Tuck-Muller et al. 2000). DNA hypomethylation was found to be particularly severe in pericentromeric satellite sequences or other DNA repeat elements, and several cancers including Wilms tumor, ovarian and breast carcinomas frequently contain unbalanced chromosomal translocations with break points in the pericentromeric DNA of chromosome 1 and 16 (Qu et al. 1999; Ehrlich 2009; Yoshida et al. 2011). Hypomethylation of satellite sequences might predispose them to breakage and recombination. Jaenisch and colleagues showed that neurofibromatosis 1 $(Nf1)^{+/-}$ $Trp53^{+/-}$ mice were 2.2 times more prone to loss of heterozygosity (LOH) when a hypomorphic *Dnmt1* allele was introduced (Eden et al. 2003). Similarly, hypomethylation of L1 retrotransposons promote chromosomal rearrangements in colorectal cancer (Suter et al. 2004; Ogino et al. 2008). LINE1 hypomethylation has been implicated in cancers of bladder, ovary, liver and colon (Kim et al. 2009; Dammann et al. 2010; Schernhammer et al. 2010; Wilhelm et al. 2010). The mechanism behind global hypomethylation in cancers largely remains unknown but several experiments point towards the involvement of SWI/SNF chromatin-remodeling complexes. Individuals with the developmental disorder ATRX (α-thalassaemia, myelodysplasia) have mutations in the *ATRX* gene, which encodes a SNF2-family helicase. In mutant ATRX cells, the ribosomal DNA repeats are hypomethylated (Gibbons et al. 2000). *Lsh*, a SNF2-family member is required for maintenance of normal DNA methylation as its knockout leads to global defect in genomic methylation and chromosomal instability (Fan et al. 2003). A common splice variant of the *de novo* DNA methyltransferase-*DNMT3B* (*DNMT3b4*) that was identified in patients with liver cancer, is also associated with hypomethylation of pericentromeric satellite sequences (Saito et al. 2002). Further, it was shown that mice carrying a hypomorphic allele for the maintenance methyltransferase *Dnmt1* (which reduces expression of Dnmt1 to 10% of wild type levels), developed tumors early and showed chromosomal instability (Gaudet et al. 2003). Hypomethylation of CpG islands has been implicated in overexpression of *CYCLIND2* and *MASPIN* in gastric carcinoma, *MN/CA9* in human renal cell carcinoma, *S100A4* metastasis-associated gene in colon cancer and human papillomavirus 16 (*HPV16*) in cervical cancer (Nakamura and Takenaga 1998; Cho et al. 2001; Badal et al. 2003; De Capoa et al. 2003; Lacobuzio-Donahue et al. 2003; Oshimo et al. 2003; Piyathilake et al. 2003; Sato et al. 2003).

Global DNA hypomethylation is associated with progression of multiple types of cancers including cervical, ovarian, multiple myeloma, chronic lymphocytic leukemia and breast cancer (Cho et al. 2010; Missaoui et al. 2010; Fabris et al. 2011; Walker et al. 2011). In case of gastric, tongue and esophageal carcinogenesis, global DNA hypomethylation was negatively correlated with invasiveness (Baba et al. 2009; Tomita et al. 2010).

26.3.2.2 Gene-Specific Hypermethylation

Aberrant transcriptional silencing of genes associated with DNA hypermethylation of their promoter region is probably the most intensely studied epigenetic abnormality in cancers. It is difficult to answer as to why certain genes become methylated during carcinogenesis, but several hypotheses have been proposed to address this issue. The Darwinian theory suggests the selective growth advantage conferred to cells upon inactivation of particular genes as the reason why they become methylated in tumors (Esteller 2005). Also, it has been suggested that genes which are under the control of polycomb proteins are more vulnerable to DNA methylation (Ohm and Baylin 2007). Like DNA hypomethylation, hypermethylation can play a seminal role in neoplastic evolution. Promoter DNA hypermethylation can silence specific genes including tumor suppressor genes in cooperation with histone modifications. For example, hypermethylation was found to be associated with deacetylation of histone H3 and H4, loss of histone H3- lysine4 methylation and gain of H3K9 methylation (Baylin and Ohm 2006).

While Retinoblastoma (*RB*) was the first tumor suppressor gene shown to be silenced by DNA hypermethylation, $p16^{ink4A}$ is one of the most common tumor suppressors exhibiting loss of function following DNA hypermethylation. It exhibits DNA hypermethylation during progression of lung cancers and even in preneoplastic lesions (Belinsky et al. 1998; Nuovo et al. 1999) and its germ line loss leads to increase in hematopoietic stem cell (HSC) life span in terms of their ability for tissue maintenance and repair (Janzen et al. 2006; Krishnamurthy et al. 2006; Molofsky et al. 2006). The silencing of $p16^{ink4A}$ gene has also been reported in preinvasive stages of breast, colon and other cancers. Furthermore, experimental loss of $p16^{ink4A}$ appears to facilitate early tumorigenesis by being permissive for subsequent emergence of genomic instability (Kiyono et al. 1998) and may directly allow for additional epigenetic silencing of other genes (Reynolds et al. 2006). Germ line mutations of many tumor suppressors cause familial forms of cancers. The same tumor suppressor genes have been found to be promoter DNA hypermethylated in subsets of non familial cancers such as *VHL* in renal, *APC* in colon and *BRCA1* in breast cancers (Ting et al. 2006a, b). Till date, a large number of tumor suppressor or candidate tumor suppressor genes have been identified to be DNA hypermethylated in multiple cancer types, including but not limiting to: *p53, RASSF1A, p14ARF, CDKN2A, p16, p21, TIMP3, ECRG4, HIC1* (Cohen et al. 2003; Amatya et al. 2005; Chanda et al. 2006; Gotze et al. 2009; Juhlin et al. 2010; Dadkalos et al. 2011; Radpour et al. 2011).

Tumor suppressors are not the only genes to exhibit DNA hypermethylation; a multitude of other genes like *hMLH1, MGMT, E-CADHERIN, CALCITONIN*, etc. are silenced in cancers (Esteller 2007; Jacinto and Esteller 2007). Apart from individual gene hypermethylation, in some cancers, groups of genes were found to exhibit increased DNA methylation levels. Two such examples identified recently in cancers are, CpG island methylator phenotype (CIMP) and Long Range Epigenetic Silencing (LRES). CIMP defines a group of cancers with a 3–5 fold elevated frequency of aberrant gene methylation especially in case of *INK4A, MLH1* and *THBS1* apart from harboring microsatellite instability (Toyota et al. 1999). The clustering pattern suggestive of CIMP has been confirmed in glioblastoma, gastric cancer, liver cancer, pancreatic cancer, esophageal cancer, ovarian cancer, acute lymphocytic leukemia and acute myelogenous leukemia apart from colorectal cancer (Issa 2004). Though the causes leading to CIMP are largely unknown, its presence is associated with a poor outcome in multiple malignancies (Issa 2003). Age-related methylation, life-style, exposure to epimutagens, chronic inflammations have all been thought of as important contributors of CIMP. Long range epigenetic silencing (LRES) is a phenomenon where large regions of chromosomes can be coordinately suppressed. Typically, LRES can span megabases of DNA and involve broad heterochromatin formation accompanied by the hypermethylation of clusters of contiguous CpG islands within the region. This process is usually associated with DNA and histone hypermethylation and can in turn lead to DNA methylation of flanking, non-methylated genes. Such hypermethylated regions spanning hundreds of kilobases involving gene family clusters have also been found in esophageal, neuroblastoma, breast and colorectal cancers (Frigola et al. 2006; Clark 2007).

26.3.3 The DNA Methyltransferases

DNA methyltransferases (DNMTs) are the enzymes, which catalyze the covalent addition of methyl groups to cytosine in the CpG dinucleotide context. Broadly they have been classified into: maintenance methyltransferases (*DNMT1*) and *de novo* methyltransferases (*DNMT3A, DNMT3B, DNMT3L*). *DNMT2*, with its weak methyltransferase activity has not been classified into either of the groups. The DNMTs are characterized by a 'C' terminal catalytic domain, which transfers methyl group from S-Adenosyl-L-methionine (SAM) to cytosine and contains five conserved amino acid motifs, namely I, IV, VI, IX and X. Motifs I and X form the AdoMet binding site.

Dnmt1 was the first methyltransferase to be identified. Homologs of *Dnmt1* have been found in nearly all eukaryotes that have DNA bearing 5-methylcytosine, but not in species that lack 5-methylcytosine. It is a large protein of 1,620 amino acids, having an 'N' terminal regulatory region containing an NLS, a region that targets *Dnmt1* to replication foci, PHD like domain, proliferating cell nuclear antigen (PCNA)-binding domain apart from the 'C' terminal catalytic domain. An 'N' terminal truncated but enzymatically active, oocyte-specific isoform of *Dnmt1*

(*Dnmt1o*) has also been identified, which accumulates to high levels in the cytoplasm of embryos (Doherty et al. 2002). Being a maintenance methyltransferase, Dnmt1 is involved in the bulk of DNA methylation and has also been implicated in non-CpG methylation (Grandjean et al. 2007). The DNMT3 family comprises *de novo* methyltransferases: *DNMT3A, DNMT3B* and an enzymatically inactive paralogue *DNMT3L*. Both DNMT3A and DNMT3B have a regulatory 'N' terminal domain containing an ATRX-like Cys-rich domain (PHD domain) and a PWWP domain, which are involved in interaction of these enzymes with other proteins and in targeting them to heterochromatin (Gowher et al. 2005). DNMT3L on the other hand doesn't possess any catalytic activity owing to mutations within all the conserved motifs that contain the catalytic residues of DNA-(cytosine-C5)-methyltransferase. It interacts with and regulates the *de novo* methyltransferases DNMT3A and 3B, stimulating their activity (Chedin et al. 2002; Suetake et al. 2004; Kareta et al. 2006). Recently, it has also been shown that DNMT3L interacts with Histone 3 Lysine 4 (H3K4) when it is unmethylated, providing a link between *de novo* DNA methylation and histone modifications (Ooi et al. 2007).

Homozygous deletion of Dnmts: *Dnmt1, Dnmt3a, Dnmt3b* are lethal in mice (Li et al. 1992; Lei et al. 1996; Okano et al. 1999). The deletion of *Dnmt3a* and *Dnmt3b* abolishes *de novo* methylation, while *Dnmt1* depletion leads to bulk DNA demethylation (Okano et al. 1999). Embryos of *Dnmt1*$^{-/-}$ mice are stunted, show delayed development, and do not survive past mid-gestation (Li et al. 1992). Though *Dnmt3a*$^{-/-}$ homozygous mutant mice developed to term and appeared normal at birth, they became runted and died at about 4 weeks of age (Okano et al. 1999). On the other hand, *Dnmt3b*$^{-/-}$ homozygous mutant mice were not viable, though they appeared to develop normally till E 9.5. These embryos displayed multiple developmental defects including growth impairment and rostral neural tube defects (Okano et al. 1999). Similarly, *Dnmt3a*$^{-/-}$ and *Dnmt3b*$^{-/-}$ double homozygous embryos were smaller in size and showed abnormal morphology at E 8.5, E 9.5 and died before E 11.5. A closer analysis of the embryos revealed that their growth and morphogenesis were arrested shortly after gastrulation (Okano et al. 1999).

Conditional deletion of *Dnmt3b* was also shown to result in DNA hypomethylation leading to chromosomal instability and spontaneous immortalization in mouse embryonic fibroblasts (Dodge et al. 2005). The DNMT3 family also plays an important role in genomic imprinting. Pericentromeric satellites are one of the specific targets of *DNMT3B*. Furthermore, inactivation of *DNMT3B* in humans leads to ICF syndrome, which is associated with low methylation in pericentromeric satellite regions. *Dnmt3a* conditional mutant females died *in utero* and lacked methylation and allele-specific expression at many maternally imprinted loci (DMRs of *Snrpn, Igf2r, Peg1*), whereas *Dnmt3a* conditional mutant males showed impaired spermatogenesis and lacked methylation at a few paternally imprinted loci examined in spermatogonia (*H19* DMR, *Dlk1-Gtl2* IG-DMR) (Kaneda et al. 2004). DNMT3A2, a germ-cell-specific isoform of *DNMT3A* is also required for genomic imprinting.

Though enzymatically inactive, DNMT3L is required for DNA methylation in ES cells where it is expressed in high amounts compared to differentiating somatic

cells (Ooi et al. 2010). Mice obtained after disruption of *Dnmt3l* by targeted mutation were viable and of normal phenotype in both heterozygous and homozygous conditions (Bourc'his et al. 2001). The male progeny however were found to be sterile and displayed azoospermia as their spermatocytes failed to complete meiosis, apart from exhibiting decrease in methylation at the differentially methylated regions of *H19* and *Rasgrf1* imprinted genes (Webster et al. 2005). On the other hand, the heterozygous progeny of homozygous females died before mid gestation (at 9.5 days post coitum) and these embryos were found to lack methylation imprints at *Snrpn* and *Peg1* imprinted genes apart from other pre-natal growth defects (Bourc'his et al. 2001). The role of Dnmt3l in establishing patterns of DNA methylation for several imprinted genes during gametogenesis has also been established by other groups (Webster et al. 2005; Arima et al. 2006).

26.3.4 The DNA Demethylase Enzymes

In contrast to the well characterized DNA methyltransferases, the enzymes that catalyze the removal of methyl moiety from methylcytosine have remained enigmatic. It has been easy to envisage passive mode of demethylation, wherein cytosine methylation is lost upon replication without being followed by maintenance methylation of hemimethylated DNA. However, active demethylation observed in the zygote (specifically for the paternal genome) and during germ cell formation has been hard to explain (Mayer et al. 2000; Ooi and Bestor 2008; Wu and Zhang 2010). Only very recently, a few proteins have been identified that modify methylcytosine either to thymine or hydroxymethylcytosine, which can be directly or indirectly converted to cytosine by DNA repair enzymes (Morgan et al. 2004; Rai et al. 2008; Kriaucionis and Heintz 2009; Tahiliani et al. 2009; Ito et al. 2010).

Conversion of methylcyotsine to thymine in mammals is catalyzed by Apolipoprotein B RNA-editing catalytic component-1 (Apobec-1) and Activation Induced Deaminase (AID, also known as Apobec2), members of the cytidine deaminase family (Morgan et al. 2004; Rai et al. 2008). The role of these enzymes in active demethylation of methylcytosine has been confirmed by various experiments directly or indirectly. In 2004, it was demonstrated that AID had 5-methylcytosine deaminase activity *in vitro* (Morgan et al. 2004). Moreover, it was found that AID is expressed in primordial germ cells (PGCs), oocytes and cells from early mouse embryos, cells which are known for their active demethylation activity (Morgan et al. 2004; Wu and Zhang 2010). That AID is indeed involved in DNA demethylation during PGC specification was confirmed by Popp et al. (2010) when they showed that erasure of DNA methylation mark during PGC formation is hindered in AID deficient mice. The role of AID in DNA demethylation was further strengthened by the observation that AID deficiency prevents the demethylation of pluripotency specific genes (*OCT4, NANOG*) during the process of converting fibroblasts into pluripotent iPS cells (Bhutani et al. 2010).

It was recently discovered that TET family of proteins including TET1, TET2 and TET3 can convert methylcytosine into hydroxymethylcytosine (Tahiliani et al. 2009; Ito et al. 2010). JBP1 and JBP2 proteins catalyze conversion of methyl-thymine into β-D-glucosyl-hydroxymethyluracil in Trypanosomes. TET proteins were identified to be their homologs in mammals based on iterative sequence profile computational search (Tahiliani et al. 2009). This study also demonstrated that cytosine does exist in the hydroxmethylcytosine form in the mammalian genomes (also shown by Kriaucionis and Heintz 2009) and TET1 does convert methylcytosine to hydroxymethylcytosine *in vitro* (Tahiliani et al. 2009). Like Activation induced deaminase, TET proteins mediated conversion of methylcytosine to hydroxymethylcytosine has also become established as a part of the DNA demethylation process. TET1 has been shown to be involved in maintaining ES cell pluripotency and in the specification of inner cell mass during embryogenesis (Ito et al. 2010). Just after fertilization and before the first cell division, the male pronucleus has been shown to get actively demethylated (Mayer et al. 2000). A very recent study showed that unlike the previous established notion, methylcytosines in male pronucleus gets converted into hydroxymethylcytosine and not cytosine (Iqbal et al. 2011). Moreover, conversion back to cytosine is not an active process as hydroxymethylcytosine stays in the genome even after several cell divisions (Iqbal et al. 2011). The same paper also indicated on the basis of transcriptional profiling that TET3 rather than TET1 was probably responsible for the conversion to hydroxymethylcytosine (Iqbal et al. 2011).

26.3.5 DNMTs and Cancer

Being the effectors of DNA methylation, DNMTs have an important role to play in the aberrant DNA methylation observed in cancer cells (Fig. 26.2). DNMT protein levels and activities were found to be elevated in various cancer types, including gastric, bladder, brain, leukemia, colon and lung (Issa et al. 1993; Belinsky et al. 1996; Melki et al. 1998; De Marzo et al. 1999; Robertson et al. 1999; Ramsahoye et al. 2000; Girault et al. 2003; Li et al. 2003; Xiong et al. 2005; Amara et al. 2010; Qu et al. 2010). Moreover, multiple DNMTs have been found to co-localize to promoters of hypermethylated genes and also have been defined as components of the transcriptional repression complexes (Di Croce et al. 2002; Kim et al. 2002; Datta et al. 2003). However, it has been difficult to correlate the increased expression of DNMTs with CpG hypermethylation in several cancers (Miremadi et al. 2007). While the maintenance methylation activity of DNMT1 is primarily observed in somatic cells, the *de novo* methylation activities are seen in germ cells and at embryonic stages. This paradigm of separation of methylation activity has been challenged in the cancer setting.

Though aberrant DNA methylation patterns observed in cancer is an undisputed fact, it is still not clear as to which of the DNMTs are primarily responsible. The maintenance methyltransferase *DNMT1* has been implicated because its expression

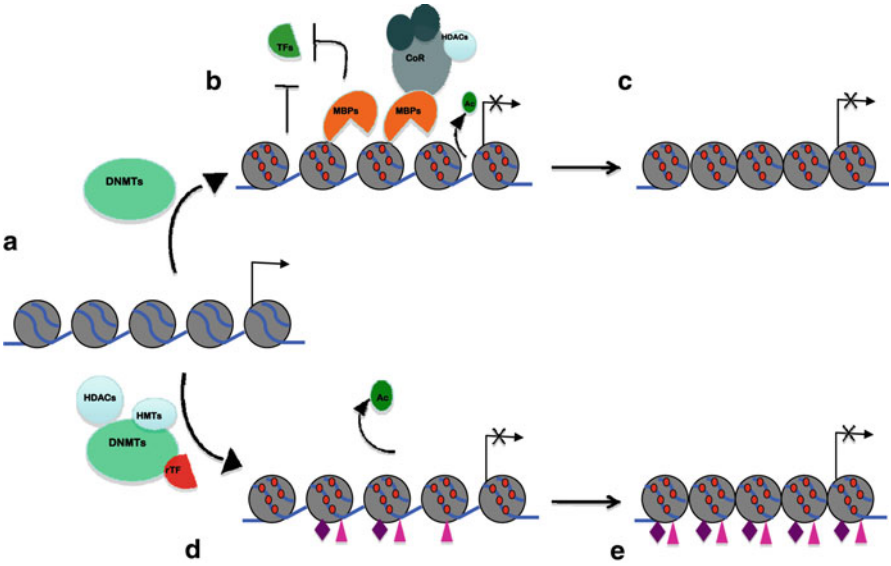

Fig. 26.2 Dual role of DNMTs in silencing of gene expression. *DNMTs* can be targeted directly or with the help of transcriptional repressors (*rTF*) to CpG dinucleotides. (**a**) Nascent chromatin strand (**b** & **c**) DNMTs can get directly targeted to a genomic locus due to a stimulus like hemi-methylated DNA and lead to CpG methylation. This could cause inhibition of transcription factor (*TF*) binding either directly or due to the binding of *MBPs* (Methyl binding proteins). The MBPs can recruit co-repressor complexes (*CoR*) and *HDACs* leading to nucleosomal remodeling and chromatin compaction. (**d** & **e**) The DNMTs are also found in complex with histone deacetylase and histone methyltransferases. In this scenario DNA methylation is coupled to setting up of repressive histone marks including H3K9me3 and H3K27me3 eventually leading to condensed and tightly packed chromatin. *Raised arrows* denote transcriptional activation; *crossed arrows* denote transcriptional repression. *Red circles*: Methyl CpG; *Green circles*: H3K9 acetylation; *Circles* with *curved lines* denote nucleosomal organization of chromatin; *Diamonds* represent H3K9me3; *Triangles* represent H3K27me3

levels are higher in cancers and it also exhibits low levels of *de novo* methylation activity against unmethylated substrates (Jair et al. 2006). DNMT1 overexpression resulted in detectable *de novo* methylation of CpG island in human fibroblasts (Vertino et al. 1996) and induced transformation in NIH3T3 cells (Wu et al. 1993). On the other hand, severe depletion of DNMT1 produced only minor decrease in overall methylation, minimal loss of promoter hypermethylation and undetectable re-expression of silenced tumor suppressor genes in colorectal cancer cells (Rhee et al. 2000, 2002; Ting et al. 2004, 2006a, b). However, a double knock out of *DNMT1* and *DNMT3b* (*DNMT1*$^{-/-}$ *DNMT3b*$^{-/-}$) in HCT116 cells resulted in >95% loss in genomic methylcytosine content and complete promoter demethylation apart from re-expression of aberrantly silenced genes (Rhee et al. 2002; Akiyama et al. 2003; Suzuki et al. 2004). *DNMT1* is thus one candidate that is capable of initiating aberrant CpG island hypermethylation in cancer cells. Indeed, DNMT1 has been

reported to repress transcription through its interactions with methyl CpG binding proteins, histone deacetylases and histone methyltransferases (Bogdanovi and Veenstra 2009). On the other hand, global methylation levels were affected indirectly through the regulation of DNMT1 by BRCA1 and miRNA29b (Garzon et al. 2009; Shukla et al. 2010). In the Apc^{Min} mice model, a reduction of *Dnmt1* activity, due to heterozygosity of *Dnmt1* gene, in conjugation with treatment using DNMT inhibitor like 5-Aza-cytidine reduced the average number of intestinal adenomas (Laird et al. 1995). Similar observations in genetically engineered mice involving *Dnmt1* clearly demonstrate a causal relationship between alteration of DNA methylation and cancer. But the requirement of the maintenance methyltransferase *DNMT1* in maintaining promoter hypermethylation and gene silencing in cancer cells is still debatable. Mutational inactivation of *DNMT1* has not been observed in most of the cancers though colorectal cancers infrequently harbored these mutations (Kanai 2008). Among the other DNMTs, germ line single nucleotide polymorphisms (SNPs) in *DNMT3B* have been associated with risk of breast cancer, gastric cancer, hepatocellular carcinoma, lung adenocarcinoma and lung cancer (Cebrian et al. 2006; Ezzikouri et al. 2009; Hu et al. 2010).

Recently data from our own laboratory showed loss of methylation at *DNMT3L* promoter in cervical cancer and few other cancer types (Gokul et al. 2007; Manderwad et al. 2010 and unpublished results). This decrease in methylation was associated with an increased expression of DNMT3L. Moreover, overexpression of DNMT3L in HeLa cells lead to increased cell proliferation and anchorage-independent growth in a time and passage dependent manner (Gokul et al. 2009). Microarray analysis revealed that the expression patterns of genes important in nuclear reprogramming, development and cell cycle were misregulated. Interestingly, among this misregulated gene set, many imprinted genes were found to be downregulated; consistent with the role of DNMT3L in imprinting. DNMT3L was also identified to be essential for the growth of human testicular germ cell tumors (Minami et al. 2010).

26.3.6 DNA Demethylase Enzymes and Cancer

Though the role of AID in DNA demethylation has been identified only recently, its role in creating somatic hypermutations and in class-switch recombination of human immunoglobulin genes has been known for some time now (Revy et al. 2000; Marusawa and Chiba 2010). Several studies have reported unregulated expression of AID in cancer (Matsumoto et al. 2007; Klemm et al. 2009). An interesting correlation that has been investigated is the link between *Helicobacter pylori* infection, aberrant expression of Activation Induced Deaminase and gastric cancer (Matsumoto et al. 2010; Touati 2010; Endo et al. 2011). A few studies have also highlighted the role of aberrant AID expression in causing widespread genomic instability (Klemm et al. 2009; Robbiani et al. 2009).

TET1 is an abbreviation for Ten-Eleven Translocation-1 and was named so because of its involvement in a t(10;11)-associated leukemia (Lorsbach et al. 2003).

It was simultaneously identified by Ono et al. (2002) who found *TET1* to be fused to *MLL* gene in Acute Myeloid Leukemia. Later studies have also reported fusion of *TET1* (also known as *LCX*) with *MLL* suggesting a role for *TET1* in carcinogenesis (Shih et al. 2006; Burmeister et al. 2009, reviewed in Dahl et al. 2011). The fusion of *TET1* and *MLL* is also interesting from an epigenetic perspective as MLL itself is H3K4 histone methyltransferase (Krivtsov and Armstrong 2007). Deletions and mutations have also been reported for *TET2* in several myeloid cancers (Figueroa et al. 2010; Ko et al. 2010; Langemeijer et al. 2011). Along with the mutations in TET proteins, a recent report showed that the levels of hydromethylcytosine were also altered in myeloid cancer (Ko et al. 2010) indicating that the process of DNA demethylation itself might be key to the process of carcinogenesis.

26.3.7 DNA Methylation Binding Proteins and Cancer

The transcriptional repressive activities of DNA hypermethylation are primarily interpreted and mediated by a family of proteins which harbor the methyl-CpG-binding domain (MBD) – the protein motif responsible for binding methylated CpG dinucleotides (Bird and Wolffe 1999; Bird 2002; Burgers et al. 2002). These include MECPs (Methyl CpG binding protein-MECP2), MBDs (Methylcytosine-binding protein MBD1, 2, 3, 4) and the novel protein Kaiso (Prokhortchouk et al. 2001). Except for MBD3, the other methyl binding proteins specifically recognize methyl-CpG (Klose and Bird 2006). These proteins can mediate silencing of gene expression by recruiting other members of the epigenetic machinery, primarily the chromatin remodeling co-repressor complexes (Jones et al. 1998; Ng et al. 1999; Wade et al. 1999; Zhang et al. 1999; Sarraf and Stancheva 2004). The MBDs have a high specificity towards gene promoters and they have been found at hypermethylated and aberrantly silenced cancer gene loci (Ballestar and Esteller 2005; Ting et al. 2006a, b). For example, MBD2 binds to aberrantly methylated promoter of tumor suppressor genes: *p14/ARF* and *p16/ink4A* in colon cancer cell lines (Magdinier and Wolffe 2001; Martin et al. 2008) and suppress their expression. Importantly, MBDs are found to be associated with complexes that contain HDACs; for example the methyl binding proteins, MECP2, MBD1 and MBD2 have been found to associate with transcriptional co-repressors, such as SIN3A, which are known to bind HDACs directly (Jones et al. 1998; Ng et al. 1999). This binding results in compaction of chromatin and stable repression of the target gene. MBD1 interacts with histone H3 methyltransferase (SETDB1) linking epigenetic marks on DNA to histone modifications, whereas MBD4 is thought to act as a thymine DNA glycosylase, repairing G:T or G:U mismatches at CpG sites (Kanai 2008). The newly identified methyl binding protein- Kaiso, associates with the histone deacetylase containing-N-CoR co-repressor complex bringing about repression of methylated genes (Yoon et al. 2003). Interestingly, mice lacking Kaiso have been shown to exhibit resistance to intestinal tumorigenesis (Prokhortchouk et al. 2006). Polymorphisms (SNPs) in many of these MBD proteins (MBD1, MBD2 and MBD4) were found to be associated

with an increased risk of cancer (lung and breast). Overexpression of MeCP2 has been observed in breast cancer (Muller et al. 2003) and it is implicated in the silencing of *IL-6* in pancreatic adenocarcinoma cell lines (Dandrea et al. 2009). MeCP2 also plays important roles in gastric and colorectal carcinogenesis (Pancione et al. 2010; Wada et al. 2010). Progeny of a cross between Mbd2-deficient mice and $Apc^{Min/+}$ mice were found to be resistant to the development of intestinal tumors and this resistance was dependent on the dosage of Mbd2 (Sansom et al. 2003). The role of MBD2 has also been positively implicated in the silencing of Nrf2 expression in a mouse prostate adenocarcinoma model (Yu et al. 2010). The hypermethylated region of human telomerase reverse transcriptase (*hTERT*) was also shown to be specifically associated with MBD2 bringing about its transcriptional repression in many cancer cell lines (Chatagnon et al. 2009).

26.3.8 DNA Methylation Inhibitors and Epigenetic Therapy

In spite of being robust, epigenetic modifications like DNA methylation and histone modifications are reversible in nature. This characteristic of epigenetic modifications makes them a useful target for cancer therapy and the enzymes that mediate and maintain DNA methylation (DNMTs) and various histone modifications (HATs, HDACs and HKMTs) are the prime drug targets in the epigenetic therapy proposed for cancer.

Various small molecules have been identified with inhibitory effects on DNA methylation, histone methylation or acetylation and it is possible to reactivate a hypermethylated tumor suppressor gene by the use of DNA demethylating agents or DNA methyltransferase inhibitors (DNMTi). Most of these molecules are nucleoside analogs, which are incorporated into replicating DNA in place of cytosine. Once incorporated, they can sequester DNMTs by the formation of a covalent bond between them, eventually depleting the cell of enzymatic activity resulting in heritable demethylated DNA (Juttermann et al. 1994; Santi et al. 1984). Due to the high replicative potential of cancer cells they are particularly prone to demethylation underscoring the efficacy of DNMTi as antineoplastic drugs. Among the best studied inhibitors are 5-aza-cytidine and 5-aza-2′-deoxycytidine which were found to bring about DNA demethylation (Sorm et al. 1964, reviewed in Momparler and Bovenzi 2000; Gal-Yam et al. 2008; Kwa et al. 2011). Both inhibitors were shown to reactivate genes silenced by aberrant methylation in various human tumor cell lines (Momparler and Bovenzi 2000) apart from inducing in vitro differentiation of human leukemic cells (Pinto et al. 1984; Momparler et al. 1985). They also exhibited potent antineoplastic activity in hematological malignancies and lung cancer (Rivard et al. 1981; Pinto and Zagonel 1993; Momparler et al. 1986; Momparler and Ayoub 2001). Presently, of the four epigenetic-based drugs that have been approved by FDA for cancer therapy, two are inhibitors of DNMTs: 5-azacytidine (Vidaza) and 5-aza-2′-deoxycytidine (Decitabine) (Kwa et al. 2011). Both drugs have been approved by the FDA for the treatment of myelodysplastic syndrome, a preleukemic disease (Pinto

and Zagonel 1993). There are few other inhibitors like Epigallocate, Procaine etc. some of which are less toxic compared to 5-aza-cytidine, but their dosage, stability and efficacy have impeded their advancement to clinical trials (Kwa et al. 2011). Zebularine is another promising DNMTi which has the advantage of higher stability and longer half-life together with the convenience of oral administration but is effective only in high doses (Champion et al. 2010; Cheng et al. 2004; Kwa et al. 2011). Recently, derivatives and variants of 5-azacytidine like S110 and 2′-deoxy-5,6-dihydro-5-azacytidine have been characterized and found to be less toxic and more stable demethylating agents (Chuang et al. 2010; Matousova et al. 2011). In spite of concerns relating to non-specific side effects, toxicity, drug resistance and inefficacy against solid tumors, DNMTis remain an attractive choice as therapeutic agent in hematological malignancies. Additionally because of their ability to cross the blood brain barrier they can be used to treat brain malignancies (Diede et al. 2010).

The interplay between histone modifications and DNA methylation in cancer cells has prompted the use of histone deacetylase inhibitors (HDACi) with DNMTi. Such combinatorial therapy was shown to increase the expression of silenced genes to higher levels than with either of the inhibitors alone (Issa 2007; Kwa et al. 2011). Cameron et al. (1999) were the first to show the synergistic transcriptional activation of the *p16CDKN2A* tumor suppressor gene upon treatment of tumor cells with 5-azacytidine and Trichostatin A, a HDACi. We in our lab also have been able to show similar 5-azacytidine transcriptional activation for the *DNMT3L* gene in cervical cancer cells upon treatment with 5-azacytidine and Trichostatin A (Gokul et al. 2009). It was also shown that the treatment of tumor cells with 5-azacytidine decreased levels of repressive histone marks (H3K9me3) while increasing active histone marks (H3K4me3) at promoters of genes that are aberrantly silenced by DNA methylation (Nguyen et al. 2002). These facts not only underscore the importance of such combinatorial therapy but also warrant the need to investigate the nature of synergy between different inhibitors to bring about an optimal response in tumors.

26.4 Concluding Remarks

The abnormalities in cancer change the “epigenetic landscape” of the cell and involve multiple aberrations in virtually every component of the epigenome. Since epigenetic silencing processes are mitotically heritable, they play the same roles and undergo the same selective processes as genetic alterations in the development of cancer. Similar to genetic mutations, epigenetic events follow the Darwin’s hypotheses for the evolution of species by which alterations in gene expression induced by epigenetic events which confer cellular growth advantage are selected for in the host, resulting in the progressive, uncontrolled growth of the tumor. Can epigenetic mechanisms initiate tumorigenesis by their own? As described in this chapter there are numerous examples of aberrant epigenetic changes occurring in various cancers as early events. For example, DNA hypomethylation in cancers has been shown to be an early event, but it has not been conclusively shown nor are there examples that

show epigenetic processes initiating cancer on their own. However, the evidence accumulated over the last two decades reiterates the complexity of the mechanisms involved in cancer development and emphasizes the fact that there is strong interplay between epigenetic and genetic events in cancer and that understanding the epigenetic basis of carcinogenesis is critical in solving the puzzle that cancer is.

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Chapter 27
Role of Epigenetics in Inflammation-Associated Diseases

Muthu K. Shanmugam and Gautam Sethi

Abstract There is considerable evidence suggesting that epigenetic mechanisms may mediate development of chronic inflammation by modulating the expression of pro-inflammatory cytokine TNF-α, interleukins, tumor suppressor genes, oncogenes and autocrine and paracrine activation of the transcription factor NF-κB. These molecules are constitutively produced by a variety of cells under chronic inflammatory conditions, which in turn leads to the development of major diseases such as autoimmune disorders, chronic obstructive pulmonary diseases, neurodegenerative diseases and cancer. Distinct or global changes in the epigenetic landscape are hallmarks of chronic inflammation driven diseases. Epigenetics include changes to distinct markers on the genome and associated cellular transcriptional machinery that are copied during cell division (mitosis and meiosis). These changes appear for a short span of time and they necessarily do not make permanent changes to the primary DNA sequence itself. However, the most frequently observed epigenetic changes include aberrant DNA methylation, and histone acetylation and deacetylation. In this chapter, we focus on pro-inflammatory molecules that are regulated by enzymes involved in epigenetic modifications such as arginine and lysine methyl transferases, DNA methyltransferase, histone acetyltransferases and histone deacetylases and their role in inflammation driven diseases. Agents that modulate or inhibit these epigenetic modifications, such as HAT or HDAC inhibitors have shown great potential in inhibiting the progression of these diseases. Given the plasticity of these epigenetic changes and their readiness to respond to intervention by small molecule inhibitors, there is a tremendous potential for the development of novel therapeutics that will serve as direct or adjuvant therapeutic compounds in the treatment of these diseases.

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27.1 Introduction

The term epigenetics was first coined by developmental biologist Conrad H. Waddington in 1942, is defined as heritable changes in gene expression patterns that occur without any changes in the primary DNA sequence but changes that are sufficient to regulate the pattern of gene expression. Recently, this definition has been elaborated to include transient changes in gene expression (Hirst and Marra 2009). Epigenetic regulation plays a pivotal role in development and differentiation though all cells of an organism have the same DNA sequence, they can differentiate into a multitude of diverse cell types (Feinberg 2007). It is now becoming increasingly apparent that the epigenetic changes together with genetic abnormality drives chronic inflammatory diseases that ultimately lead to tumor progression, and that cancer is the manifestation of both genetic and epigenetic modifications (Egger et al. 2004; Esteller 2008). The word inflammation is derived from the Latin word "inflamacio" denoting to 'set a fire'. The classical signs of inflammation are redness (rubor), swelling (tumor), heat (calor) and pain (dalor). The first four classical signs were described by the Roman physician Cornelius Celsus (ca. 30 BC–38 AD), while loss of function was added by a German physician Rudolf Virchow in 1870. Virchow postulated that microinflammation that results from irritation leads to the development of most chronic diseases including cancer. It was Rudolf Virchow who first linked inflammation with cancer, atherosclerosis, diabetes, arthritis, obesity, allergy, and other chronic diseases (Heidland et al. 2006). In naming the pathological conditions, inflammation is usually indicated by adding the suffix '-itis', such as bronchitis, esophagitis, gastritis, colitis, pancreatitis, prostitis, cervicitis, and hepatitis. Some conditions such as asthma and pneumonia, although inflammatory conditions, do not follow this convention (Aggarwal and Gehlot 2009). Since Virchow proposed a connection between inflammation and cancer, it has been estimated that approximately 15% of all cancers are linked to inflammation, including chronic inflammatory bowel disease and colorectal cancer (Balkwill and Mantovani 2001), associations between cervical cancer and the human papillomavirus (HPV); liver cancer and hepatitis B or C virus (Saigo et al. 2008); Barrett's esophagus, and esophageal cancer; and that inflammation alone will not cause cancer; mutations and epigenetic events from environmental exposures or alterations in immunity are also key contributors in the cancer process (Schottenfeld and Beebe-Dimmer 2006). Several proinflammatory mediators, such as cytokines, chemokines, prostaglandins (PGs), nitric oxide (NO) and leukotrienes disrupt normal signaling cascades within cells which contributes to the development of neoplasms (Surh et al. 2005). The primary defense response initiated by the human body is the activation of immune system upon injury via recruitment of circulating cells such as macrophages, monocytes, lymphocytes, neutrophils and leukocytes. The activated cells thus act on the injured site and reduce the inflammation in an acute attack. In cases of severe inflammation, these cells are in turn activated, leading to excessive production of proinflammatory molecules. This phenomenon leads to the process of chronic inflammation which has been implicated in the development of various major human diseases including malignancies (Aggarwal et al. 2006). The importance of the epigenome in the

pathogenesis of common human diseases is likely to be almost as significant as that of traditional genetic mutations (Portela and Esteller 2010). The enzymes that are involved in these epigenetic changes are also being currently used as targets for drug development (Eliseeva et al. 2007; Selvi et al. 2010b; Wagner et al. 2010).

Eukaryotic DNA is wrapped around an octamer of the core histones H2A, H2B, H3, and H4, thus building the fundamental unit of chromatin, the nucleosome (Berger 2007). Posttranslational modifications of the histone tails and cytosine methylation of the DNA determine the accessibility of the chromatin and, therefore, the ability of transcription factors to bind and initiate gene expression (Berger 2007). These chromatin marks are unstable and they rapidly change in response to any external stimulus (Berger 2007; Bird 2007) and any permanent changes to the DNA can lead to development of defective organs and in the development of diseases. This primarily happens at specific dinucleotide sites along the genome, i.e., cytosines 5′ of guanines, or at CpG sites. In fact, 40% of genes contain CpG-rich islands upstream from their transcriptional start site, and up to 70–80% of all CpG dinucleotides in the genome are methylated (Bird 2002; Wilson 2008). Aberrant DNA methylation has been observed with inflammation, viral infection, in cancer and DNA methylation pattern has been shown to be molecular marker for diagnosis of early cancer (Esteller 2008). It is also related to the effectiveness of therapeutic agents affecting DNA methylation and histone deacetylation. Epigenetic modifications of chromatin and DNA have been recognized as important accommodating and expressive or suppressive factors in controlling the expression of gene transcription (Wilson 2008). Two major epigenetic mechanisms are the posttranslational modification of histone proteins in chromatin and the methylation of DNA itself, which are regulated by distinct pathways (Wilson 2008). The epigenome is influenced by environmental factors throughout life. Nutritional factors can have profound effects on the expression of specific genes by epigenetic modification. Despite global hypomethylation, distinct CpG islands located in regulatory regions of genes can be specifically hypermethylated, leading to a repressed transcription of the associated genes (Das and Singal 2004). Differences in methylation status of CpG sites, monoallelic silencing and other epigenetic regulatory mechanisms have been constantly observed in key inflammatory response genes (Portela and Esteller 2010; Wilson 2008).

Recent literature review strongly supports the hypothesis that a number of critical genes such as those involved in cell cycle control CDKN2 (Otterson et al. 1995), apoptosis DAPK1 (Katzenellenbogen et al. 1999), DNA repair BRCA-1 (Dobrovic and Simpfendorfer 1997), metastasis TIMP-3 (Kang et al. 2000), drug resistance, differentiation, and angiogenesis are silenced by epigenetic modification processes, such as histone hypermethylation. Thus, methylation of CpG dinculeotides is a common biological mechanism for switching off gene expression, and more interestingly, this is not only limited to cancer. While DNA mutations have been closely linked with chronic inflammation driven cancers in certain individuals, evidence also suggests that epigenetic alterations can also contribute to familial cancer risk (Fleming et al. 2008). Autoimmune and neoplastic diseases increase in frequency with increasing age, with epigenetic dysregulation proposed as a potential explanation. Chronic inflammation has long been recognized as instrumental to cancer progression (Brigati et al. 2002; Coussens and Werb 2002), and recent data linking inflammation to initiation

of variety of diseases can be explained on the basis of epigenetics dysregulation (Mantovani et al. 2008; Rosin et al. 1994). Another recent study has clearly indicated epigenetic interplay between histone modifications and DNA methylation in gene silencing (Vaissiere et al. 2008) and the selective silencing of the DNA methyltransferase B has been correlated to the acute fatty-acid-induced non-CpG methylation of proliferator-activated receptor gamma (PPAR-gamma) coactivator-1 alpha promoter (Barres et al. 2009). Such modifications could restrain the number of targets that inhibit progression of the process of acute inflammation after an inflammatory insult. Thus, the focus of the current chapter is to highlight the evidences that epigenetic modulation may play a critical role in regulation of various inflammatory mediators and in the development of various chronic inflammation driven diseases.

27.2 Epigenetic Modifications on Tumor Necrosis Factor-α gene

Tumor necrosis factor (TNF-α) was first isolated as an anticancer cytokine more than two decades ago, however, its major role appears to be in early inflammatory responses and primarily mediates acute phase response after an inflammatory insult (Aggarwal 2003; Balkwill 2009; Sethi et al. 2008). TNF-α is secreted by a variety of cells that infiltrate the site of injury such as neutrophils, monocytes, macrophages, lymphocytes and leukocytes upon stimulation of Toll like receptors by lipopolysaccharide (Foster et al. 2007), or activation via cytokines and lipid mediators (Hayes et al. 1995; Lee and Sullivan 2001; Lin et al. 2004; Takeda and Akira 2004). Promoter hypomethylation of the Toll-like receptor 2 (TLR2) gene in bronchial epithelial cells leads to pro-inflammatory response when stimulated with bacterial peptidoglycan (Shuto et al. 2006) and in intestinal epithelial cells epigenetic modifications regulate TLR4 (Takahashi et al. 2009). DNA methylation and histone acetylation play an important role in establishing epigenetic modification across *TNF-α* locus (Sullivan et al. 2007). However, when dysregulated and secreted in the circulation, TNF-α can mediate the development of a wide variety of diseases, including cancer (Aggarwal 2003; Balkwill 2009; Szlosarek et al. 2006). Epigenetic modifications of the *TNF-α* locus occur both developmentally and in response to acute stimulation and, importantly they actively regulate expression by increase in DNA methylation (Kruys et al. 1993). The *TNF-α* locus migrates from heterochromatin to euchromatin in a progressive fashion, reaching euchromatin slightly later in differentiation. Finally, histone modifications characteristic of a transcriptionally competent gene occur with myeloid differentiation and progress with differentiation (Sullivan et al. 2007). Additional histone modifications characteristic of active gene expression are acquired with stimulation. In each case, manipulation of these epigenetic variables altered the ability of the cell to express TNF-α. MLL, a histone methyltransferase has been shown to bind to *TNF-α* promoter and ATF2 and CBP, both histone acetylators have been implicated in the transcriptional regulation of *TNF-α* and H3 lysine 4 methylation appears to be important for transcription (Hess 2004) (Dou et al. 2006; Guenther et al.

2005; Hayakawa et al. 2004; Kang et al. 2007; Pogribny et al. 2007; Tsai et al. 2000). However, the pathways responsible for activation of these molecules differ from cell type to cell type. Majority of the studies are done on myeloid cells as they produce the bulk of TNF-α. Maturation of monocytic cells and high glucose environment have been shown to increase histone acetylation at the *TNF-α* locus and in turn leads to increased TNF-α expression (Lee et al. 2003; Miao et al. 2004). Inter-individual variation in TNF-α production with upto 40-fold differences has been explained by polymorphisms in the *TNF-α* promoter region (Wilson et al. 1997). For example, *TNF-α*-308A allele was associated with more severe outcomes in various infectious diseases (McGuire et al. 1994). In another set of experiments, the methylation status of the *TNF-α* promoter (−310 to +30) was examined in LPS-stimulated human macrophages. This region of the *TNF-α* promoter contains 12 CpG dinucleotides. Lower methylation at two specific CpG sites (−304 and −245) was correlated with high production of TNF-α mRNA, suggesting that the methylation state of the *TNF-α* promoter may be an important factor in driving the level of *TNF-α* gene expression, and it may help to explain the origins in variation in the inflammatory response between individuals (McGuire et al. 1994). In addition, *TNF-α* gene in murine monocytic cell line exhibit significant increase in histone acetylation (Ramirez-Carrozzi et al. 2006) and studies on other cytokines have indicated that tissue specific expression may be controlled epigenetically and that histone modification represents a common regulatory strategy for cytokines (Lee et al. 2006). In contrast, inhibition of histone deacetylases causes increase in global histone acetylation increased the ability of the cells to secrete TNF-α (Ramirez-Carrozzi et al. 2006). These findings may have relevance for inflammatory disorders in which TNF-α is overproduced. The proinflammatory role of TNF-α has been linked to all steps involved in tumorigenesis, including cellular transformation, survival, proliferation, invasion, angiogenesis, and metastasis (Sethi et al. 2008). TNF-α overexpression has been observed in variety of inflammation driven diseases such as a rheumatoid arthritis, Crohn's disease, ulcerative colitis and asthma. Recently, Set7/9 was shown to play an important role in the manifestation of inflammatory disorders where TNF-α is over-expressed (Li et al. 2008). These studies demonstrate the importance of epigenetic regulation in the control of TNF-α expression. Defining the complete role of epigenetic regulation of TNF-α may lead to novel therapeutic strategies for the treatment of various chronic inflammatory diseases.

27.3 Epigenetic Changes in Interleukin genes

Histone acetylation by the lysine acetyltransferases (KATs) activates inflammatory gene transcription while increase in histone deacetylase activity leads to inhibition of inflammatory gene transcription (Duncan et al. 2011). Especially in chronic inflammatory diseases increased acetylation of histones at the promoter region of inflammatory genes is mediated by NF-κB. Inflammatory interleukins overexpression

has been linked with the development of diseases, which suggests that inflammation plays a major role in disease progression (Bayarsaihan 2011). Proinflammatory interleukins include IL-1, IL-2, IL-6, IL-8, and IL-12. IL-1α, expressed in both normal tissue and several tumor cells, is a regulatory cytokine that can induce the activation of transcription factors, including NF-κB and AP-1, and promotes the expression of genes involved in cell survival, proliferation, and angiogenesis (Wolf et al. 2001). Histone H4 hyperacetylation is a well known inflammation associated epigenetic mark. IL-1β causes histone acetylation on H4 at K8 and K12 residues (Ito et al. 2000) and has also been reported to rapidly acetylate histone tails mediated by CBP/p300 (Villagra et al. 2010). In contrast, HDAC recruitment leads to histone deacetylation and gene repression. HDACs regulate the transcription of both pro and anti-inflammatory genes by recruiting co-repressor complexes GATAs, ZEB1, and FOXp3 (Villagra et al. 2010). Age dependent changes in immune response increases the risk of infection, promote inflammation associated disease progression. Age associated hypomethylation of the DNA has been proposed as a major cause of chronic inflammation and cancer (Agrawal et al. 2010). Recently, age-dependent upregulation of IL-23p19 gene expression associated with H4K4 methylation was observed in dendritic cells (El Mezayen et al. 2009). In T cells, IL-2 gene demethylates shortly after activation leading to IL-2 production (Bruniquel and Schwartz 2003). In the differentiation of naïve CD4 T cells to T-helper 2 cells is due to rapid H3 acetylation at the IL-4/-13 gene cluster (Baguet and Bix 2004). Thus interleukins regulated inflammatory signaling pathways are either activated or silenced in inflammation driven diseases and are likely candidates that play critical role in the progression of disease to a chronic stage.

27.4 Epigenetic Changes in Oncogenes

Oncogenes also called proto-oncogenes are altered versions of normal cellular genes that play crutial role in the regulation of cell growth (Croce 2008; Weinberg 1994). The discovery of oncogenes, almost 30 years ago, has provided a critical breakthrough in our understanding of the molecular and genetic basis of cancer. Oncogenes have also provided important knowledge concerning the regulation of normal cell proliferation, differentiation, and apoptosis (Croce 2008). Recent reports suggest that Myc oncogene regulates complex inflammatory program (Meyer and Penn 2008) and Myc activation in B cells rapidly induces production and release of IL-1β (Shchors et al. 2006). Pleiotropic effects of oncogenes also include the induction of a pro-tumor microenvironment, through the persistent promotion of an inflammatory milieu (Borrello et al. 2008; Croce 2008; Grivennikov and Karin 2010). Approximately 20 transforming oncogenes (including ras, raf, myc, c-src, EGFR, HER-2) and a large number of tumor suppressor genes (e.g., p53, VHL, PTEN) are now known to directly trigger angiogenesis (Huang et al. 2007; Rak et al. 2000a) by up-regulation of the vascular endothelial growth factor (VEGF) in cancer cells

expressing mutant ras oncogene (Grugel et al. 1995; Rak et al. 1995). It is interesting to note that such pro-angiogenic effects can be mimicked and/or amplified by exposing tumor cells to bona fide epigenetic stimuli such as hypoxia (Harris 2002; Laderoute et al. 2000; Mazure et al. 1996; Semenza 2000), hypoglycemia (Semenza 2000), inflammatory cytokines (Cohen et al. 1996) hormonal stimulation (Jain et al. 1998), and altered cell-cell contact (Koura et al. 1996; Rak et al. 2000b; Sheta et al. 2000). Global hypomethylation of genomic DNA has been identified as a participant in the aggressive behavior of cancer cells by upregulating oncogenes such as c-Myc or h-Ras (Das and Singal 2004). Promoter hypomethylation can activate the aberrant expression of oncogenes and induce loss of imprinting (LOI) in some loci. For example, MASPIN (also known as SERPINB5), a tumor suppressor gene that becomes hypermethylated in breast and prostate epithelial cells (Futscher et al. 2004), appears to be hypomethylated in other tumor types. MASPIN hypomethylation, and therefore its expression, increases with the degree of dedifferentiation of some types of cancer cells (Bettstetter et al. 2005; Futscher et al. 2002). Thus, the complex interplay between the histone methylation and DNA methylation and the specificity in epigenetic mechanisms that regulate DNA methylation pattern still remains to be elucidated in detail.

27.5 Epigenetic Changes in the Regulation of Master Transcription Factor NF-κB

The transcription factor nuclear factor-kappa B (NF-κB), discovered by David Baltimore in 1986, is present in the nucleus and binds the promoter of immunoglobulin kappa chain in B cells (Sen and Baltimore 1986, 2006). In the mammalian cells, the NF-κB family of transcription factors is composed of homodimers and heterodimers derived from five distinct subunits, RelA (p65), c-Rel, RelB, p50 (NF-κB1) and p52 (NF-κB2). All family members share a highly conserved Rel homology domain (RHD; ~300 aa) responsible for DNA binding, dimerization domain, and interaction with IκBs, the intracellular inhibitor of NF-κB (Karin 2006; Sethi et al. 2008; Sethi and Tergaonkar 2009). In unstimulated cells, the majority of NF-κB complexes are kept predominantly cytoplasmic and in an inactive form by binding to a family of inhibitory proteins, the IκBs (Karin 2006; Sen and Baltimore 2006). Generally, the inactive NF-κB/IκBα complex is activated by phosphorylation on two conserved serine residues within the N-terminal domain of the IκB proteins. Phosphorylation of these conserved serine residues in response to stimulation, leads to the immediate polyubiquitination of IκB proteins by the SCF-β-TrCP complex (Karin 2006). This modification subsequently targets IκB proteins for rapid degradation by the 26S proteasome (Karin 2006). Activation of the NF-κB signaling cascade can result in phosphorylation and degradation of IκBα, allowing translocation of NF-κB to the nucleus, where it induces gene transcription (Ahn and Aggarwal 2005; Ahn et al. 2007; Shen and Tergaonkar 2009;

Vallabhapurapu and Karin 2009). NF-κB is activated by many divergent stimuli, including proinflammatory cytokines (e.g., TNF-α, IL-1β), T- and B-cell mitogens, bacteria, lipopolysaccharide (LPS), viruses, viral proteins, double-stranded RNA, and physical and chemical stresses (Sethi and Tergaonkar 2009). Activated NF-κB binds to specific DNA sequences in target genes, designated as κB elements, and regulates transcription of over 400 genes involved in inflammation, immunoregulation, tumor cell proliferation, invasion, metastasis, angiogenesis, chemoresistance and radioresistance (Li and Sethi 2010; Mantovani 2010; Wong and Tergaonkar 2009).

In chronic inflammation activation of inflammatory response is mainly mediated through NF-κB which is regulated by acetylation, lysine methylation and arginine methylation (Cheung et al. 2007). The main modification is acetylation which has been shown to be important regulators of inflammatory gene expression (Medzhitov and Horng 2009) in T-cells and monocytes (Lal et al. 2009; Wells 2009; Wierda et al. 2010). Acetylation of distinct lysine residues of RelA regulates NF-κB transcriptional activation, DNA binding affinity, IκB-α assembly and subcellular localization (Chen et al. 2001; Chen and Greene 2003). IKK-α has been shown to bind to NF-κB dependent promoter via polymerase II complex and CREB binding protein (CBP), where it can acetylate histone H3 on Lys9 (Yang et al. 2008) and also can phosphorylate histone H3 at Ser10 (Anest et al. 2003). This IKK-α dependent and cytokine mediated phosphorylation is critical in CBP-mediated acetylation of histone H3 on Lys14 (Yamamoto et al. 2003). Acetylation of histone H3 is often seen in cytokine mediated inflammation and in autocrine and paracrine activation that follows with increased NF-κB driven inflammatory gene expression (Barnes 2009a). Glucocoticoids and HDAC2 can reverse NF-κB driven inflammation (Barnes 2009b). Along with NF-κB poly (ADP-ribose) polymerase-1 (PARP-1) has been show to play prominent role in chronic inflammation driven diseases (Aguilar-Quesada et al. 2007). PARP-1 has been demonstrated as a promoter specific co-activator of NF-κB *in vivo* and independent of its enzymatic activity (Hassa et al. 2003). PARP-1 directly interacts with p300, p50 and p65 and synergistically co-activates NF-κB dependent transcription (Hassa et al. 2005). Under inflammatory conditions, p300 can in turn acetylate PARP-1 at specific lysine residues in variety of cell types. NF-κB mediated activation of histone H3, H4 acetylation by IL-1β, TNF-α, or endotoxins can increase the expression of granulocyte-macrophage colony stimulation factor (GM-CSF)(Aggarwal 2004). Thus reversible acetylation of NF-κB and proteins like PARP-1 and histones play a major role in regulating gene expression during inflammation. Altered methylation pattern such as lysine methylation are also observed in inflammatory diseases. H3K4 methyltransferase SET7/9 can also regulate recruitment of NF-κB-p65 to promoters and thereby modulate inflammatory gene expression (Li et al. 2008). Arginine methylation has not been directly implicated in inflammation; however, PRMT1 and PRMT4 are known transcriptional co-activators of NF-κB (Covic et al. 2005; Hong et al. 2004; Meyer et al. 2007). In hyperglycemic memory, high transcriptional activity of pro-inflammatory transcription factor NF-κB is due to enzymatic deletion of H3K9 methyl marks from its promoter (Brasacchio et al. 2009; El-Osta et al. 2008) and this finding provides novel perspective to the link between NF-κB activity and epigenetic modification in

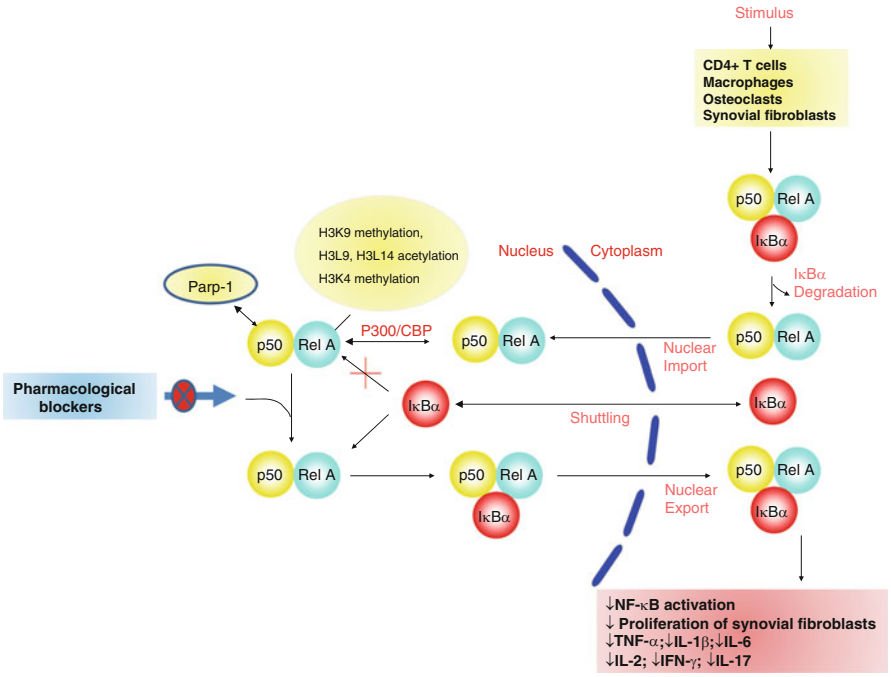


Fig. 27.1 Histone methylation and acetylation mediated aberrant NF-κB transcriptional activation in tumor cells. Epigenetic activation in the gene promoter region and interactions between DNA methylation machinery, chromatin modifiers (HDACs) and PARP-1. Pharmacological inhibition of individual components in the repressive complex with DNMT inhibitors and HDAC inhibitors, either alone or in combination, result in DNA demethylation and deacetylation and complex disintegration leading to reversal of genome-wide epigenetic alterations in cancer cells through reversal of NF-κB mediated gene transcription and cell signaling pathways

the development of chronic inflammation driven diseases (Taylor 2008; Villeneuve et al. 2008). Overall, epigenetic modifications in NF-κB as described briefly in Fig. 27.1 and associated inflammatory molecules leads to the development of major inflammatory diseases which will be now be discussed in detail below.

27.6 Epigenetic Changes During Autoimmune Diseases

Rheumatoid arthritis (RA) is a chronic inflammation driven autoimmune disease of the joints that occurs in 1% of world population (Firestein 2003). Rheumatoid arthritis arises from interplay of inherited genetic predisposition such as HLA-DR allele subtypes and specific gene polymorphism, autoantibody production and dietary factors (Ermann and Fathman 2001; Smith and Haynes 2002). One of the hallmarks of autoimmune disorders such as rheumatoid arthritis is the infiltration of

circulating inflammatory cells and synovial fibroblasts. Synovial inflammation is mainly caused by overproduction of cytokines, chemokines and matrix metalloproteinases that eventually lead to the progressive destruction of articular cartilage and bone (Ospelt and Gay 2008). Several studies have shown that treatment of RA with inhibitor of TNF-α or IL-6 confirmed that the disease is driven by differences in cytokine production (Feldmann and Maini 1999). By analyzing DNA methylation status in circulating T-cells of RA patients, severe hypomethylation was observed in the T-cells of RA patients compared to healthy T-cells (Neidhart et al. 2000; Richardson et al. 1990). In addition, Nile et al. evaluated the DNA methylation status in the promoter region of IL-6 gene (−1,200 to +30) in PBMC cells of RA patients. They found that hypomethylation at a single CpG site at −1,181 contributed to elevated expression of IL-6 gene which contributes to sustained inflammatory condition (Nile et al. 2008). Sullivan et al. investigated DNA methylation of CpG-rich sequences within the promoter of the TNF-α gene and found a positive correlation between high expression levels of TNF-α and low methylation status (McInnes and Schett 2007). Epigenetic histone modifications in RA have mostly concentrated on histone acetylation and particularly on the use of HDAC inhibitors as therapeutic agents. There have been a number of reports of beneficial effects from the use of HDAC inhibitors *in vitro* and *in vivo* (Grabiec et al. 2008). Intravenous administration of HDI FK-228 reduces the expression of TNF-α and IL-1β in a mouse model of autoantibody-mediated arthritis (AMA) (Nishida et al. 2004). Prophylactic administration of SAHA and MS-275 has been shown to reduce arthritis score, radiologic score, and bone resorption in the collagen-induced arthritis (CIA) model in mice and rats (Lin et al. 2007). Trichostatin A was shown to reduce the clinical scores of arthritis and synovial inflammation in mouse AMA with concomitant decrease in expression of matrix-degrading enzymes (Nasu et al. 2008). Autoantibodies generated against citrullinated proteins are biomarker proteins and can be found in about 80% of the RA patients (Zendman et al. 2006). Therefore, studying the citrullination of proteins is of major interest in RA. PAD4 is expressed in different cell types of the RA synovium, as well as in blood monocytes (Chang et al. 2005; Vossenaar et al. 2004). Recently, Chang et al. have reported increased expression of PAD4 in RA synovial membranes compared to OA (Chang et al. 2009). However, it is still unclear to what extent PAD4 contributes to epigenetic changes involved in the pathogenesis of RA. In addition by studying the methylation pattern of death receptor 3 (DR3) in synovial cells of RA and OA patients, Takami et al. found significant hypermethylation of CpG dinuclotides in the synovial cells derived from RA. This phenomenon might explain the resistance to apoptosis seen in synovial cells of RA patients (Ashkenazi and Dixit 1998; Takami et al. 2006). Thus, in rheumatoid arthritis, the reduced activity of HDACs plays a key role in regulating NF-κB–mediated gene expression (Huber et al. 2007b). Patients with type 1 diabetes also present a characteristic pattern of histone marks, showing lymphocytes but not monocytes with increased H3K9me2 in a subset of genes associated with autoimmune and inflammatory pathways (e.g., CLTA4, IL6) (Miao et al. 2008).

27.7 Epigenetic Changes in Chronic Inflammation Driven Cancers

Epigenetic changes have been observed in all types of cancers, understanding the regulatory mechanisms that cause severe aberrant induction of DNA methylation is gaining importance. Chronic inflammation, aging and viral infections have been shown to methylate on-core regions in CpG islands of tumor suppressor genes such as in retinoblastoma (Feinberg and Tycko 2004; Ohtani-Fujita et al. 1993), in VHL (Herman et al. 1994), CDKN2A(p16) (Gonzalez-Zulueta et al. 1995; Merlo et al. 1995), CDH1 (E-cadherin) (Graff et al. 1995; Yoshiura et al. 1995) and hMLH1 (Kane et al. 1997) that permanently repress downstream gene expression (Ahuja et al. 1998; Hsieh et al. 1998; Issa et al. 1994, 2001; Jones and Baylin 2002; Kang et al. 2002; Osawa et al. 2002). These aberrant methylation patterns provide an excellent opportunity to be exploited as tumor biomarkers and as targets for chemotherapeutics (Egger et al. 2004; Issa et al. 2004; Laird 2003). There are many comprehensive reviews on the role of epigenetics and cancer (Feinberg and Tycko 2004; Jaenisch and Bird 2003; Jones and Baylin 2002; Laird 2003). Here in this chapter we will discuss the role of epigenome in inflammation driven cancer development. These changes occur in genes involved in cell cycle progression, angiogenesis, matrix adhesion molecules and metastasis thus leading to cancer development. In cancer, the most fundamental characteristic is the heritable changes such as gene silencing due to CpG hypermethylation at the promoter regions of tumor suppressor genes. Loss of tumor suppressor proteins, for eg., RB1, VHL, p16INK4A, MLH1, BRCA1, APC, st7 and nm23 have been shown to disrupt almost all cell signaling pathways that promote cancer development (Hanahan and Weinberg 2000; Pal et al. 2004). Genes such as RB1, VHL, and BRCA1 hypermethylation is restricted to hereditary cancers such as retinoblastoma, renal, breast and ovarian cancer (Dobrovic and Simpfendorfer 1997; Herman et al. 1994; Stirzaker et al. 1997; Yang et al. 2006a). In contrast p16INK4A is mutated in the germline of patients with hereditary malignant melanoma (Pho et al. 2006) and pancreatic cancers (Goldstein 2004), and is often inactivated by promoter hypermethylation in a wide variety of cancers, including breast, gastrointestinal, respiratory tract, gynecological, and hematopoietic cancers (Aniello et al. 2006; Chevillard-Briet et al. 2002; Frietze et al. 2008; Gronbaek et al. 2000; Kondo et al. 2008; Li et al. 2006; Luo et al. 2006; Majumder et al. 2006; Wang et al. 2004; Yang et al. 2006a). Agents that inhibit arginine methylation and histone deacetylation uphold chromatin integrity and prevent chromatin condensation and reestablish demethylation and gene transcription (Cheng et al. 2004; Verbiest et al. 2008). Thus histone modifying enzymes have a critical role in determining the tumor progression and multidrug resistant phenotype of cancer cells. A list of various histone modifying enzymes associated with cancer development is summarized in Table 27.1. In particular DNA methylation and histone modifications are very attractive anti-cancer targets towards development of novel therapeutic approaches (Gronbaek et al. 2007).

Table 27.1 A list of major histone modifying enzymes linked to the development of cancer

Histone methyl transferases/acetylase	Enzymes linked to various cancers	References
Arginine methyl transferases	PRMT1, PRMT2, PRMT4, PRMT5, RPMT7, CARM1	Cheung et al. (2007), Chevillard-Briet et al. (2002), Frietze et al. (2008), Hong et al. (2004), Meyer et al. (2007), Majumder et al. (2006), Pal et al. (2004), Verbiest et al. (2008)
Lysine methyl transferases	MLL1 (KMT2A), MLL4 (KMT2D), EZH2 (KMT6), SUV39H1/2 (KMT1A/B), G9a (KMT1C), Eu-HMTase1 (KMT1D), SETDB1/ESET (KMT1E), SET8/PR-SET7 (KMT5A), SMYD2 (KMT3C), SMYD3, DOT1L (KMT4)	Kang et al. (2007), Pogribny et al. (2007), Aniello et al. (2006), Hess (2004), Kondo et al. (2008), Li et al. (2006), McGarvey et al. (2006), Saigo et al. (2008), Bachmann et al. (2006), Bryant et al. (2007), Collett et al. (2006), Ding et al. (2006), Hamamoto et al. (2004, 2006), Huang et al. (2007), Okada et al. (2005), Shi et al. (2007)
Histone demethylases	LSD1; JHDM1; JMJD2C (GASC1); JMJD6; PADI4	Agger et al. (2008), Chang et al. (2007), Cloos et al. (2006), Metzger et al. (2005), Shi et al. (2004), Tsukada et al. (2006), Wissmann et al. (2007), Cuthbert et al. (2004)
Histone acetyltransferase and histone deacetylases	HATs; HDAC1, HDAC2, SET7 (HMT), SirT1	Portela and Esteller (2010)

27.8 Epigenetic Changes During Chronic Obstructive Pulmonary Disease

Chronic obstructive pulmonary disease (COPD) is a chronic inflammatory disease of the lung which leads to the development of lung cancer (Bowman et al. 2009; Lee et al. 2009; Yao and Rahman 2009) Lymphocytes, macrophages, and neutrophils are found in abundant at the site of inflammation and are the main orchestrators and amplifiers in the progression of COPD. However, these inflammatory cells can be manipulated by immunosculpting and immunoediting pathways in the progression of cancer, and immuno-based therapeutic strategies for cancer to induce immune escape of cancer cells especially in a tumor-promoting microenvironment which is due to chronic inflammation seen in lungs of patients with COPD (Reiman et al. 2007). Steroid resistance observed in COPD patients have been attributed to decreased expression of HDAC in lungs of patients with COPD (Ito et al. 2005). Chromatin remodeling is manifested by post-translational modifications of core histone proteins and DNA methylation which is shown to regulate proinflammatory gene expression during the development of COPD and

lung carcinogenesis. High levels of histone acetylation is observed on the promoters of proinflammatory genes in alveolar macrophages and airway epithelial cells in patients with COPD, and the extent of acetylation is significantly correlated with disease severity (Ito et al. 2005). The fundamental mechanism of hyperacetylation of histones/nonhistone proteins in lungs of patients with COPD is associated with reduced HDAC2 level (Adenuga et al. 2009; Ito et al. 2005). This phenomenon is also observed in lungs of rodents exposed to cigarette smoke (Adenuga et al. 2009; Yang et al. 2006b). Increasing HDAC2 activity or levels by phenolic antioxidants and theophylline, are being tested as therapeutic strategies to reduce the lung inflammatory response and attenuates corticosteroid resistance in patients with COPD (Barnes 2009a). Methylation of p16 promoter has been detected in the sputum of patients with COPD and is correlated with heavy cigarette smoking (Adenuga et al. 2009). Genome-wide DNA demethylation with site-specific hypermethylation occurs in lung cancer cells and is associated with silencing of a variety of tumor-suppressor genes by the recruitment of HDACs (Barnes 2009b). The mechanisms underlying these observations may be due to aberrant expression/activity of DNA methyltransferases (DNMTs) and demethylases in cancer cells. Methylation in the promoters of multiple genes is shown in adenocarcinomas and in non -small cell lung cancer (NSCLC), and this methylation is associated with tumor progression and recurrence (Brock et al. 2008). Therefore, determination of DNA methylation on specific gene may provide the useful biomarkers for early detection and/or chemoprotective intervention in lung cancer. Modifications of core histone proteins increase the complexity of epigenetic alterations mediated by aberrant DNA methylation in cancer cells. Increased HDAC1, and decreased HDAC5 and HDAC10 are correlated with advanced stage of disease and adverse outcome in lung cancer patients (Barnes 2009a). DNA demethylating agents and HDAC inhibitors synergistically induce apoptosis in lung cancer cells, and prevent lung cancer development in animals exposed to tobacco carcinogens (Adenuga et al. 2009). However, the specificity on a particular isoform of HDACs, optional therapeutic doses, timing, and mode of administration are still under evaluation for these agents.

27.9 Epigenetic Changes Observed During Neurodegenerative Disorders

Aberrant epigenetic changes have also been observed in neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease and Huntington's disease and in neurological disorders such as epilepsy, multiple sclerosis and in amyolotropic lateral sclerosis which are primarily driven by chronic inflammation (Portela and Esteller 2010). Alzheimer's disease originates from the production of amyloid β (Aβ) via the proteolytic cleavage of amyloid precursor proteins (APP) by presenilin 1 and presenilin 2 play crutial roles in neurodegeneration. Neprilysin (discoidin domain receptor tyrosine kinase 1), is the major Aβ-degrading enzyme in the brain (Iwata et al. 2000) is hypermethylated in cerebral endothelial cells when these cells

are treated with high concentrations of Aβ (Chen et al. 2009). However, neprilysin does not seem to be regulated by DNA methylation in NB7 and SH-SY5Y cells, but by histone acetylation (Belyaev et al. 2009). Furthermore overexpression of APP C-terminal peptide in PC12 cells and in rat primary cortical neurons increases acetylation of histones H3K14 and H4K5 (Kim et al. 2004). In addition, neuron-specific overexpression of HDAC2 in mice, but not of HDAC1, can decrease dendritic spine density, synapse number, synaptic plasticity, and memory formation (Guan et al. 2009). S100A2 and SORBS3 genes show differences in DNA methylation in Alzheimer's disease (Hauke et al. 2009; Siegmund et al. 2007). S100A2 has been previously identified as metastatic marker in non small cell lung cancer (Bulk et al. 2009).

Parkinson's disease is characterized by progressive loss substantia nigra dopaminergic neurons and striatal projections which causes tremor, muscle rigidity, bradykinesia and postural instability(Urdinguio et al. 2009). Dopamine depletion observed in this disease is associated with decrease in histone H3K4me3 (Urdinguio et al. 2009). Chronic levodopa therapy leads to deacetylation of histones H4K5, K8, K12, and K16. MPTP (1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine), widely used in Parkinson's disease model, induces H3 acetylation, which is reduced after treatment with levodopa (Nicholas et al. 2008). Thus, epigenetic regulation in this disease might have important role, however, its direct implications remain unknown (Hauke et al. 2009). Multiple sclerosis is a chronic inflammatory disease characterized by a demyelination process followed by degeneration(Urdinguio et al. 2009). For example, hypomethylation at the promoter region of PADl2 (peptidyl arginine deiminase, type II), was also found to be overexpressed in multiple sclerosis. PADl2 catalyzes the citrullination of myelin basic protein and changes the properties of myelin (Moscarello et al. 1986). Trichostatin A is a HDAC inhibitor which has been shown to be a useful alternative treatment drug in experimental autoimmune encephalomyelitis mice. This drug has shown a reduction in spinal cord inflammation, demyelination, neuronal loss and an attenuated disability in the chronic phase (Camelo et al. 2005). It seems likely that epigenetic alteration mechanisms, such as DNA methylation and transcriptional dysregulation, are a marker of disease status in Alzheimer's disease as well as other neurodegenerative disorders.

27.10 Small Molecule Compounds as Inhibitors of Epigenetic Changes Induced by Histone Modifying Enzymes

27.10.1 HDAC Inhibitors

There is growing interest in the various mechanisms that regulate chromatin remodeling, including modulation of histone deacetylase activities (Lee et al. 2008). Evidence suggests that such epigenetic factors may be important in understanding the basic pathological variation in inflammatory response and in recent times the therapeutic potential in epigenetic manipulations in large number of chronic

inflammation driven diseases. Several groups of HDAC inhibitors have been well-characterized (Marks and Xu 2009), including short-chain fatty acids, hydroxamic acids, cyclic tetrapeptides, cyclic peptides, benzamides, and electrophilic ketones. Sodium butyrate (NaB) and valproic acid (VPA) are relatively weak short-chain fatty acid HDAC inhibitors and can inhibit histone deacetylation by non-competitive binding to classes I and IIa of HDAC-enzymes (Bolden et al. 2006; Zhang et al. 2008). HDAC inhibitors have been shown to prevent fibrosis associated with systemic sclerosis (Huber et al. 2007a), modulation apoptosis of synovial fibroblasts in RA (Jungel et al. 2006), suppression of proinflammatory cytokine production, and prevention of bone destruction in RA (Chung et al. 2003). Competitive HDAC inhibitors disrupts the cell cycle and/or induce apoptosis via upregulation of genes such as caspase3, p21 and Bax and can sensitize tumor cells to trichostatin A and polyphenol HDAC inhibitory compounds (Dashwood et al. 2006). This apparent selectivity of action in cancer cells makes HDAC inhibitors an attractive target for drug development. Liu et al. reported that the expression levels of HDAC1, HDAC3, and HDAC8 proteins were downregulated following curcumin treatment in Raji cells, whereas Ac-histone H4 protein expression was upregulated after treatment with curcumin (Liu et al. 2005). Other dietary HDAC inhibitors include sulforaphane (SFN), diallyl disulfide (DADS) and polyphenols. SFN has been reported to inhibit HDAC activity in human colorectal cancer (Myzak et al. 2004), prostate cancer (Myzak et al. 2006b) and breast cancer cells (Pledgie-Tracy et al. 2007). *In vivo* SFN retarded the growth of prostate cancer xenografts (Myzak et al. 2007) and suppressed spontaneous intestinal polyps in the Apc^{min} mouse (Myzak et al. 2006a) with evidence for altered histone acetylation status and HDAC inhibition. In general, these dietary agents are weak ligands and inhibit HDAC activity at higher concentrations than trichostatin A or SAHA, which are effective in the nanomolar to low micromolar range (Dashwood et al. 2006). There are several clinical data available for HDAC inhibitors (e.g. vorinostat and N-acetyldinaline) in the treatment of advanced NSCLC (Gridelli et al. 2008), and these agents are being investigated in randomized phase III clinical trials. In addition, synthetic histone mimetic I-BET showed anti-inflammatory activity by interfering with binding of bromodomain-containing BET proteins to acetylated histones, and disrupts the formation of the chromatin complexes essential for expression of inflammatory genes. The suppression of key inflammatory genes by I-BET suggested a potent ability of this compound to treat inflammatory conditions under *in vivo* settings (Nicodeme et al. 2010). Other HDAC inhibitors including depsipeptide and MS-275 are also currently undergoing trials for cancer treatment (Frew et al. 2009; Tan et al. 2010).

27.10.2 HAT Inhibitors

Histone acetyltransferases (HAT) have been implicated in the progression of inflammation driven diseases including various human cancers and thus represent novel, therapeutically relevant molecular targets for drug development. HAT inhibitors

obtained from purified from cashew nut shell liquid, anacardic acid, has been shown to inhibit p300 and p300/CBP-associated factor histone acetyltransferase activities (Balasubramanyam et al. 2003). Anacardic acid non-specifically inhibits p300 and PCAF with an IC50 < 10 μM (Balasubramanyam et al. 2003) Anacardic acid has also been shown to have an additive effect on apoptosis induced by TNF-α and chemotherapeutic agents like cisplatin and doxorubicin (Sung et al. 2008). A series of 28 anacardic acid analogues were prepared and tested and all the compounds showed 95% HAT inhibitory activity *in vitro* to a broad variety of cancer cell. Interestingly all the compounds tested were relatively non-toxic to normal cells (Eliseeva et al. 2007). In another study they have shown that inhibition of HAT activity by anacardic acid sensitizes cancer cells to ionizing radiation (Sun et al. 2006). Another polyphenolic compound, curcumin obtained from *Curcuma longa* inhibits p300 while it does not inhibit PCAF (Balasubramanyam et al. 2004b). Recently, a water soluble derivative of curcumin, CTK7A (Sodium 4-(3,5-bis (4-hydroxy-3-methoxystyryl)-1H-pyrazol-1-yl) benzoate) has been shown to have broad spectrum HAT inhibitory activity compared to curcumin (Arif et al. 2010). In this study, using constitutively hyperacetylated oral and liver cancer cells they showed that CTK7A can inhibit cell proliferation, attenuate HAT p300/CBP and PCAF autoacetylation while it did not affect the activity of other histone modifying enzymes like G9a, CARM1, Tip60 and HDAC1 and SIRT2 at 100 μM concentration. *In vivo* using nude mice model they examined the anti-tumor activity of CTK7A. Intra-peritoneal administration of CTK7A at dose of 100 mg/kg b.w. twice a day inhibited oral tumor growth by 50% compared to control group. This reduction in tumor growth was correlated with reduction in H3K9, H3K14 acetylation and p300 expression as determined by immunohistochemistry. Furthermore, the levels of GAPDH, NPM1, iNOS, COX2 and Ki67 levels were downregulated in CDK7A treated mice (Arif et al. 2010).

Garcinol obtained from *Garcinia indica* is a potent inhibitor of p300 acetyltransferase and PCAF (Balasubramanyam et al. 2004a). Isogarcinol, 14-isopropoxy isogarcinol and 14-methoxy isogarcinol and 13, 14 disulfoxy isogarcinol specifically inhibit p300 HAT activity but not PCAF activity (Arif et al. 2009). Most lysine acetyl transferase inhibitors possess poly-hydroxy functional groups (Selvi et al. 2010b). Plumbagin obtained from *Plumbagin ovate* and EGCG, a major phenol in green tea have been shown to inhibit p300 HAT activity (Choi et al. 2009; Ravindra et al. 2009).

27.10.3 Lysine and Arginine Methyltransferases Inhibitors

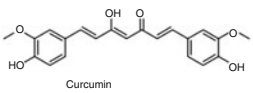
Lysine methyltransferase inhibitors represent one of the valid targets for discovery of small molecule inhibitors. A fungal derived toxin, chaeotocin was found to inhibit Su(var)3-9, a drosophila melanogaster H3K9 methyltransferase (Greiner et al. 2005). BIX-01294 is another compound that was shown to inhibit histone methyltransferas G9a and reverse H3K9 dimethylation marks in mouse ES cells and

Table 27.2 A list of epigenetic modifying enzyme inhibitors

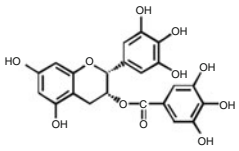
Histone modifying enzymes	Inhibitors	References
Arginine methyl transferases	AMI-1; AMI-5; Inhibitor 4b; Stilbamidine; Allantodapsone; RM-65	Cheng et al. (2004), Mai and Altucci (2009), Mai et al. (2008), Ragno et al. (2007), Selvi et al. (2010b), Spannhoff et al. (2007)
DNA methyl transferases	Azacitidine; Decitabine; Zebularine; 5-fluoro-2′-deoxycitidine; epigallocatechin-3-gallate; Hydralazine; RG108; Dietary polyphenols	Cheng et al. (2004), Link et al. (2010)
Histone deacetylases	Sinefugin; Sodium butyrate; Sodium phenylbutyrate; Valproic acid; OSU-HDAC42; Trichostatin A; Vorinostat; Panobinostat; Belinostat; Romidepsin; Entinostat; Dacinostat; Givinostat; MGCD-0103; Dietary polyphenols	Huber et al. (2010), Kuendgen and Lubbert (2008), Link et al. (2010), Vedel et al. (1978), Wagner et al. (2010)
Histone acetyltransferases	Anacardic acid; Curcumin; Curcumin analog CTK7A; Garcinol; Plumbagin; EGCG	Arif et al. (2009), Balasubramanyam et al. (2004a, b), Choi et al. (2009), Ravindra et al. (2009), Selvi et al. (2010b)
Lysine methyl transferases	Chaetocin; Bix-01294; Bix-01338	Greiner et al. (2005), Kubicek et al. (2007)

fibroblasts, but did not affect mono and trimethylation levels (Kubicek et al. 2007). In the same study, they have identified another compound that inhibits both lysine and arginine methyltransferase, BIX-01338. DZNep, 3-deazoneplanocin A, was reported to inhibit trimethylation of H3K27 and H4K20 and reactivates silenced genes in cancer (Miranda et al. 2009). Arginine methyl transferase is relatively less studied compared to other targets (Selvi et al. 2010b). The first PRMTs inhibitor was Sinefungin, was shown to inhibit myelin basic protein methylation (Amur et al. 1986). A well designed small molecule screen identified AMI-1 as a potent arginine methylation specific inhibitor with no effect on lysine methyltransferases (Cheng et al. 2004) and a carboxy analogue of AMI-1 was also found to inhibit arginine methyltransferases (Castellano et al. 2010). Ellagic acid has been shown to inhibit specifically CARM1/PRMT4 activity both *in vitro* and *in vivo* (Selvi et al. 2010a). It is know that PRMTs methylate arginine residues in variety of cancers, however, the role of arginine methylation in the initiation and progression of chronic inflammation driven diseases remains to be determined (Spannhoff et al. 2009). A list of various enzyme modifying inhibitors is summarized in Table 27.2 and the structure and plant source of natural agents that can mitigate the epigenetic process is shown in Fig. 27.2.

Turmeric
(Curcumin)



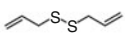
Curcumin



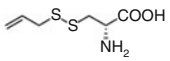
(-)-Epigallocatechin-3-gallate

Tea
(Catechins)

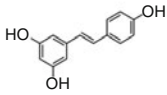
Garlic
(Diallyl sulfide, ajoene,
S-ally cysteine, allicin)



Diallyl disulfide



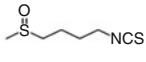
S-Allylmercaptocysteine



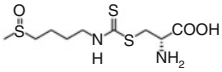
Resveratrol

Red grapes
(Resveratrol)

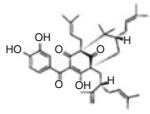
Cruciferous
vegetables
(Sulforaphane)



Sulforaphane



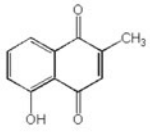
Sulforaphane cysteine



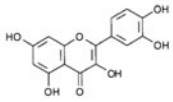
Garcinol

Garcinia indica
(Garcinol)

Plumbago ovata husk



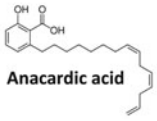
Plumbagin



Quercetin

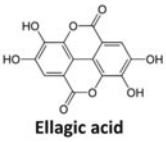
Green apples

Cashew



Anacardic acid

Raspberry



Ellagic acid

Fig. 27.2 List of small molecule from natural sources that can modulate epigenome in tumor cells

27.11 Perspectives and Conclusion

Epigenetics modulations such as DNA methylation, histone modification and knowledge about the major enzymes that mediate these alterations are central to tissue specific gene expression and development of diseases. A better understanding of these changes would provide a sound epigenetic molecular basis for various chronic inflammatory diseases. Progress in epigenetic mechanisms and alterations during inflammatory responses opens up opportunities for the development of novel HDAC and HAT inhibitors. Notable examples of HDAC inhibitor success include those of vorinostat, trichostatin A, and ITF2357 in the prevention and treatment of inflammatory diseases such as RA and different cancers. HAT inhibitors such as garcinol, anacardic acid, plumbagin, and curcumin have shown significant promise in the pre-clinical studies and well designed clinical trials are needed to validate their activities in humans. Overall, emerging evidence suggests that changes to epigenome may be important in the pathogenesis of chronic inflammation driven diseases. With advanced technology, integration of whole genome microarray technology and ChIP based screening will help in the development of novel and potent drugs that inhibit epigenetic changes with increased specificity.

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Chapter 28
Plasmodium falciparum: Epigenetic Control of *var* Gene Regulation and Disease

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Abstract *Plasmodium falciparum*, one of the deadliest parasites on earth causes human malaria resulting one million deaths annually. Central to the parasite pathogenicity and morbidity is the switching of parasite virulence (*var*) gene expression causing host immune evasion. The regulation of *Plasmodium var* gene expression is poorly understood. The complex life cycle of *Plasmodium* and mutually exclusive expression pattern of *var* genes make this disease difficult to control. Recent studies have demonstrated the pivotal role of epigenetic mechanism for control of coordinated expression of *var* genes, important for various clinical manifestations of malaria. In this review, we discuss about different *Plasmodium* histones and their various modifications important for gene expression and gene repression. Contribution of epigenetic mechanism to understand the *var* gene expression is also highlighted. We also describe in details *P. falciparum* nuclear architecture including heterochromatin, euchromatin and telomeric regions and their importance in subtelomeric and centrally located *var* gene expression. Finally, we explore the possibility of using Histone Acetyl Transferase (HAT) and Histone Deacetylase (HDAC) inhibitors against multi-drug resistance malaria parasites to provide another line of treatment for malaria.

Plasmodium, one of the most important members of Apicomplexan protozoans imposes a significant economic and health impact on human populations around the world. *Plasmodium* p*a*rasite has complex life cycle with morphologically distinct asexual and sexual developmental stages in the human host and Anopheline mosquito vector. The parasites undergo rapid transition between morphological states and antigenic variation in order to sustain chronic infection and immune evasion in human host. Parasites achieve these functions through adopting variety

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of regulatory pathways like transcriptional and posttranscriptional regulation of gene expression, translational repression and posttranslational modification of proteins. These regulatory modes help to respond to host conditions during an acute stage infection or life cycle transition of the parasite.

The word "Epigenetics" coined by Conard Waddington refers to the change and maintenance of the pattern of gene expression for generations without changing the DNA sequence of the gene (Waddington 1942; Berger et al. 2009). The epigenetic study of eukaryotic organisms helped to understand chromatin mediated regulation of gene expression. Chromatin is physiological substrate for DNA replication, repair and transcription etc. Chromatin based epigenetic mechanism involved DNA methylation, covalent and non-covalent modifications of chromatin and noncoding RNA (Goldberg et al. 2007). In *Plasmodium falciparum*, there is no experimental evidence of DNA methylation so far (Choi et al. 2006; Templeton et al. 2004). Therefore, chromatin alteration is achieved mainly through post translational modifications (PTMs) of histones, chromatin remodelling and replacement of core histones by histone variants. The presence of the several histone modifying enzymes and chromatin remodelling proteins in the parasite genome underlines the significance of epigenetics mechanism in transcriptional regulation. Therefore, epigenetic control may play a fundamental role for the parasite transcriptional regulation to overcome various critical conditions arising due to the presence of the parasites in the different hosts to complete its life cycle.

In *Plasmodium*, the epigenetic mechanism of regulation of gene expression can be divided broadly into three distinct areas based on parasite development (Merrick and Duraisingh 2010).

Firstly, during asexual intra-erythrocytic developmental stages where differential gene expression occurs which are responsible for all clinical symptoms of malaria. *Plasmodium falciparum*, the main *Plasmodium* species responsible for human mortality and morbidity shows unusual mode of gene expression during its 48 h developmental process within the erythrocyte, implying tight and integrated genome-wide regulation of transcription (Bozdech et al. 2003; Le Roch et al. 2003; Llinas et al. 2006). Recently, a battery of proteins like Api-AP2, HP1, histone deacetylases, and histone methylases have been shown to be involved in gene regulation (Balaji et al. 2005; Fan et al. 2004a, b; Iyer et al. 2008). These observations suggest the role of epigenetic mechanism in transcriptional regulation in *Plasmodium*.

Secondly, epigenetics likely play role during sexual and morphological differentiation for the rest of the life cycle. The blood stage parasites differentiate in to gametocytes. These gametocytes mate to form ookinetes followed by formation of sporozoites in the mosquito, leading to the subsequent transmission and development in the human hepatocytes before the release of the merozoites in the asexual erythrocytic cycle. Distinct transcriptional profiling has been reported in gametocytes (Yuda et al. 2009), ookinetes (Silvestrini et al. 2005), oocyst sporozoites (Raibaud et al. 2006), salivary gland sporozoites (Kaiser et al. 2004) hepatocyte stage (Matuschewski et al. 2002; Sacci et al. 2005) and erythrocyte stage (Tarun et al. 2008). All these observations may suggest epigenetic control over life cycle transition and stage differentiation.

Thirdly and most importantly, epigenetic control is involved in the mutually exclusive expression of individual *var* genes involved in the virulence processes

such as cytoadherance and variant erythrocyte invasion. The best characterized family of antigen coding gene is the *var* family in *P. falciparum*. This gene family encodes ~60 variants of *P. falciparum* erythrocyte membrane protein 1 (PfEMP1), expressed on infected erythrocytes. PfEMP1 is responsible for the attachment of the infected erythrocytes with the vascular endothelial cells thereby preventing the clearance from the circulatory system. Most of the *var* genes are generally silenced, with only one or a few being expressed at any given time (Le Roch et al. 2003; Chen et al. 1998; Duffy et al. 2002; Fernandez et al. 2002; Merrick et al. 2010; Mok et al. 2007, 2008). The switching of *var* gene expression in an allele specific manner helps parasite to evade from the host immune system leading to the chronic infection. The expressed *var* gene(s) probably does not undergo recombination, as occurs in *Trypanosoma brucei*. The *var* genes are located at the subtelomeric and internal chromosome loci and their expression patterns are marked with differential histone modifications (Scherf et al. 1998; Duraisingh et al. 2005; Freitas-Junior et al. 2005). The repression or silencing of a specific or a set of *var* genes can be reversed when the genes coding for histone deacetylase (PfSir2, HDAC1) enzymes are deleted from *Plasmodium* genome (Lopez-Rubio et al. 2007). More recently, Origin recognition complex (ORC1) protein and Heterochromatin protein1(HP1) have been suggested to have role in *var* gene regulation in *P. falciparum* (Duraisingh et al. 2005; Mancio-Silva et al. 2008; Perez-Toledo et al. 2009; Flueck et al. 2009).

Moreover, invasion proteins present in *P. falciparum*, responsible for new erythrocyte invasion are also regulated epigenetically. All these findings strongly suggest the presence of epigenetic control in *Plasmodium*.

Here we will describe the key players in epigenetic control in general, the status of them in *Plasmodium falciparum* and any significant difference from the conventional systems.

28.1 *Plasmodium* Nucleosome and Histone

The nucleosome is the building block of chromatin, consisting of ~160 bp of DNA wrapped in 1.75 superhelical turns around an octamer of core histones made of one H3/H4 tetramer and two H2A/H2B dimers (Luger et al. 1997). Histones are architectural proteins and play essential roles in DNA replication, repair and transcription. They contain five major classes H1, H2A, H2B, H3 and H4 with some gene variants. Histones H2A, H2B, H3 and H4 are the core histones and form the protein core around which nucleosomal DNA is wrapped. Each core histone contains a C-terminal tail, has a conserved histone fold domain mediating the assembly of heterodimers of specific pairs of histones. The N terminal flexible tail is accessible for several types of posttranslational modifications including phosphorylation, methylation and acetylation on serine and lysine residues, ADP-ribosylation and ubiquitination (Berger 2002). These modifications play essential roles in transcription regulation.

The malaria parasite chromosomes have a typical nucleosomal organization consisting of 155 bp of DNA (Cary et al. 1994; Lanzer et al. 1994). Nucleosomal organization extends into the telomeric repeats (TAREs) but it is totally absent at the

Table 28.1 Histones and their different modifications in *P. falciparum*

Sr. No.	Histones	Gene ID	Chromosome	Length (aa)	PTM(s)
1.	H2A	PFF0860c	11	132	N-term-ac, K3ac, K5ac
2.	H2A.Z	PFC0920w	3	158	N-term-ac, K11ac,K15ac, K19ac, K25ac K28ac, K30ac, K35ac
3.	H2B	PF11_0062	11	117	K112ub
4.	H2Bv	Pf07_0054	7	123	N-term-ac, K3ac, K8ac, K13ac, K14ac, K18ac, T85ph
5.	H3	PFF0510w	6	136	K4me, K4me2, K4me3, K9ac, K9me, K9me3, K14ac, K14me, R17me, R17me2, K18ac, K23ac, K27ac, K36me3, K56ac, K79me3
6.	H3.3	PFF0865w	6	136	K4me, K4me2, K4me3, K9ac, K14ac, R17me, R7me2, K18ac, K23ac, K27ac
7.	H4	PF11_0061	11	103	N-term-ac, R3me, R3me2, K5me, K5ac, K8ac, K12ac, K12me, K16ac, R17me, K20me, K20me2, K20me3
8.	CenH3	Pf13_0185	13	170	

telomere end. In fact, telomere is devoid of histone (Figueiredo et al. 2000). *P. falciparum* genome contains four evolutionary conserved, canonical core histones, H2A, H2B, H3 and H4 and four histone variants H2A.Z, H2Bv, H3.3 and CenH3 respectively (Miao et al. 2006; Trelle et al. 2009). In *P. falciparum*, the linker histone H1 has not been recognized suggesting higher order compaction of nuclear DNA. The details of *P. falciparum* histones are given in Table 28.1. Among the three histone H3 homologues identified, two correspond to the canonical core histone H3 and its variant H3.3, while the other resembles centromeric H3 (CenH3) (Sullivan 2003). The *P. falciparum* H3 and H3.3 protein sequences are 94% identical. PfCenH3 protein shares 61% amino acids identity with H3 and H3.3 (Cui and Miao 2010). Only one histone H4 is present in the *Plasmodium* genome and it is the most conserved among all histones (Beauchamps et al. 1997; Przyborski et al. 2003). The N-terminal tail of H4 is absolutely conserved among the different malaria parasites. Two proteins in *P. falciparum* show homology with H2B (PF07_0054 on chromosome 7 and PF11_0062 on chromosome 11 respectively) (Bennett et al. 1995). Pf11_0062 shows more homology to consensus H2B sequence than Pf07_0054 (Malik and Henikoff 2003). Therefore, Pf11_0062 is considered as Pf H2B and Pf07_0054 is referred to as PfH2Bv.

28.2 Chromosome Boundaries in *P. falciparum*

Eukaryotic genome can be differentiated in dark staining heterochromatin regions and light staining euchromatin. Heterochromatin has a tendency to spread into euchromatin regions. It has been reported that H2A.Z in yeast acts as a boundary element that

stops the spread of Sir2 mediated heterochromatin into euchromatin regions of DNA (Meneghini et al. 2003). The natural barriers to spread of heterochromatin into euchromatin are known as boundary elements. H2A.Z maintains an open configuration at promoters regions of different genes and thereby regulates transcriptional regulation of genes. In *P. falciparum*, H2A.Z co-localizes with histone modifications of euchromatin but not in heterochromatin (Petter et al. 2011). Recent reports suggest that histone variant H2A.Z in *Plasmodium* acts as a boundary element. H2A.Z is a variant of H2A and has been conserved throughout the evolution and plays crucial roles in the survival of the organism. PfH2A.Z shows enrichment in the euchromatin compartment of chromatin. In *Plasmodium* two different sirtuin proteins (PfSir2A and PfSir2B respectively) that are responsible for silencing different sets of *var* genes, have been reported. PfH2A.Z is found predominantly at the active *var* gene promoters and competes for binding sites with PfSir2A. Electron microscopy has also revealed the positioning of H2A.Z at the border between dark staining heterochromatin and light coloured euchromatin. H2A.Z deposition takes place at specific *var* gene promoters in ring stage resulting expression of *var* gene. However, in schizont stage, *var* gene promoters are devoid of H2A.Z variant. H2A.Z is the antagonist of PfSir2A but not PfSir2B because this histone variant causes expression of only PfSir2A regulated genes as shown using PfSir2A knock out parasite line where the *var* genes are found in expressed state even in schizont stage.

28.3 Histone Modifications

Canonical and variant histones contain a variety of PTMs located on the N terminal tail. The most common PTMs include acetylation, methylation, phosphorylation, ubiquitination, poly-ADP-ribosylation and sumolyation (Kouzarides 2007). Different histone modifications can alter chromatin structure by modulating the interactions of proteins with DNA and subsequently recruit effector proteins. Specific combinations of these different histone tail modifications create a "histone code" (Jenuwein and Allis 2001).

A lot of efforts have been put to identify various modification of *P. falciparum* histones using mass spectrometry (MS), liquid chromatography-tandem MS (LC-MS/MS), quadruple time-of-fight (Q-TOF) and linear trap quadruple Fourier transform (LTQ-FT). These techniques identified more than 40 post translational modifications on *P. falciparum* histones (Trelle et al. 2009) (Table 28.1).

Majority of *P. falciparum* histone modifications include histone lysine acetylation and methylation marks. Histone acetylation is linked to active genes whereas histone lysine methylation is involved in both activation and silencing. *P. falciparum* H3, H3.3 and H4 contain a number of active gene marks such as acetylated lysine and methylated lysine (H3K4) and arginines (H3R17 and H4R3) and acetylated lysine residues in H3K9. Marks for silent gene include trimethylated H3K9 and H4K20. The PfH2A.Z is acetylated on a repeated GGK motif and involved in transcriptional activation (Miao et al. 2006; Trelle et al. 2009). The PfH2Bv variant of H2B is also acetylated. For other histone modifications, H4 is found to be sumoylated,

whereas H2B is ubiquitinated at K112 (Issar et al. 2008a, b; Trelle et al. 2009). There is no evidence of histone phosphorylation yet, as is commonly observed in model eukaryotes.

Histone modifications are controlled by the various enzymes or ATP-dependent remodelers and proteins with PTM-binding modules. Below, we will describe each modification in brief, associated enzyme with that particular type of modification and the status of these modifications and enzymes in *Plasmodium*.

28.3.1 Histone Acetylation and Deacetylation

Histone lysine acetylation is catalysed by histone acetyltransferase (HATs). Five families of HATs have been reported: GNATs (**GCN5** ***N*** acetyltransferases), MYSTs (MOZ, Ybf1/Sas3, Sas2 and Tip60), p300/CBP (CREB-binding protein), general transcriptional factor HATs and nuclear hormone-related HATs (Carrozza et al. 2003; Lee and Workman 2007). At least four HATs are found in malaria parasite genomes: PF08_0034, PF11_0192, PFL1345c and PFD0795w (Horrocks et al. 2009). PfGCN5 (PF08_0034) preferentially acetylates H3K9 and K14 *in vitro* and the HAT domain can partially rescue the yeast GCN5 mutant (Fan et al. 2004a, b). The H3K9ac mark enriched in the promoters of active genes helps for gene activation. The recombinant PfMYST (PF11_0192), another HAT protein involved in the regulation of parasite cell cycle, acetylates histone H4 at K5, K8, K12 and K16 (Cui and Miao 2010). There is no evidence of HAT activity of the other two HATs.

Three classes of histone deacetylases (HDACs) have been identified in *P. falciparum*. PfHDAC1 (PFI1260c) is a class I enzyme. PF14_069 (Joshi et al. 1999) and PF10_0078 are Class II HDACs (Horrocks et al. 2009). Two class III enzymes, PfSir2A (Pf13_0152) and PfSir2B (PF14_0489) play role in regulating the mutually exclusive expression of *var* genes. PfSir2A catalyzes NAD-dependent deacetylation of acetylated lysine peptides of H3 and H4. It also shows ADP ribosyltransferase activity on all histones (French et al. 2008; Merrick and Duraisingh 2007). Both PfSir2 paralogues are required for silencing the different *var* gene promoter subsets. PfSir2A is involved in silencing of subtelomeric *var* genes transcribed towards the telomere and the internal *var* genes (promoter types *Ups*A, *Ups*E and *Ups*C), whereas PfSir2B controls *var* genes under the control of *Ups*B promoter (Tonkin et al. 2009). PfSir2A is involved in establishing heterochromatin in the subtelomeric regions and maintenance of telomeric length. Genetic deletion of either PfSir2 leads to a general derepression of subset of the *var* gene family confirming their role in *var* gene regulation (Duraisingh et al. 2005; Merrick et al. 2010; Tonkin et al. 2009).

28.3.2 Histone Methylation

Methylation is catalysed by methyltransferases that attach a methyl group onto its substrate. Bioinformatics analysis of *P. falciparum* genome reveals at least ten members of histone methyltransferases (HMTs) containing a SET [Su(var), E(z),

Trithorax] domain, characteristic of histone lysine methyl transferases. Four well characterized HKMTs (PfSET1, 2, 3 and 8) methylate H3K4, H3K36, H3K9 and H4K20 respectively (Cui et al. 2008a, b). Only recombinant PfSET2 and PfSET8 are enzymatically active and PfSET8 causes H4K20 mono-, di- and tri methylation (Cui et al. 2008a, b; Sautel et al. 2007). Malaria parasites have three arginine methyltransferases (pRMTs): PfRMT1 (PF14_0242), PfRMT5 (Pf13_0323) and PfCARM1 (PF08_0092) (Fan et al. 2009). Only PfCARM1 is well characterized. It catalyzes mono- and di-methylation of H4R3 and some nonhistone substrates (Fan et al. 2009).

The malaria parasite genome also contains two types of histone demethylases (HDMs), the lysine specific demthylases (LSD1) and JmjC (jumionji C) domain containing histone demethylases (JHDMs). There are at least one LDS1 (PFL0575w) and two JHDMs (MAL8P1.111 and PFF0135w) in *Plasmodium* (Shi and Whetstine 2007). The role of histone methylation in gene regulation and maintenance of the subtelomeric heterochromatin needs to be explored further in *P. falciparum*.

28.3.3 Histone Ubiquitination

Malaria parasites encode several proteins involved in ubiquitination pathways. These include paralogs for polyubiquitin (PFL0585w, PY03971), two ubiquitin-ribosomal protein fusions (Ub-S27a and Ub-52) (PF14 0027, PF13 0346), neural precursor cell expressed developmentally and down-regulated 8 (NEDD8) (MAL13p1.64, Pv122475), small ubiquitin-related modifier (SUMO) (PFE0285c, Pv097850), homologs to ubiquitin 1 (HUB1) (PFL1830w, Pv100840), ubiquitin-related modifier 1 (URM1) (Pf11 033, PY06420) and autophagy 8 (ATG8) (PF10 0193, Pv001860) (Ponts et al. 2008). The H2A and H2B are commonly subjected to histone ubiquitination. The H2A ubiquitination is considered as a repressive mark and H2B ubiquitination is involved in both transcriptional activation and silencing (Weake and Workman 2008). The malaria parasite genome contains a number of protein homologues of E1 (ubiquitin/UBL activating enzymes), E2 (ubiquitin/UBL conjugating enzymes) and E3 (ubiquitin/UBL ligases) (MAL8P1.23, PF08_0094, PF08_0020, PFl0470, PFB0440c etc.) enzymes and proteases that might be involved in removing Ubl modifications (Ponder and Bogyo 2007; Ponts et al. 2008). Role of histone ubiquitination in the regulation of parasite gene transcription is not known clearly. Two deubiquitination enzymes PfUCH54 (PF11_0177) and PFUCHL3 (PF14_0576) have been identified in *P. falciparum* (Artavanis-Tsakonas et al. 2006; Frickel et al. 2007).

28.3.4 Histone Sumoylation

In malaria parasites, PfSUMO proteins have been identified which sumoylate H4 (Issar et al. 2008a, b). Histone H4 sumoylation is associated with decreased gene expression (Shiio and Eisenman 2003).

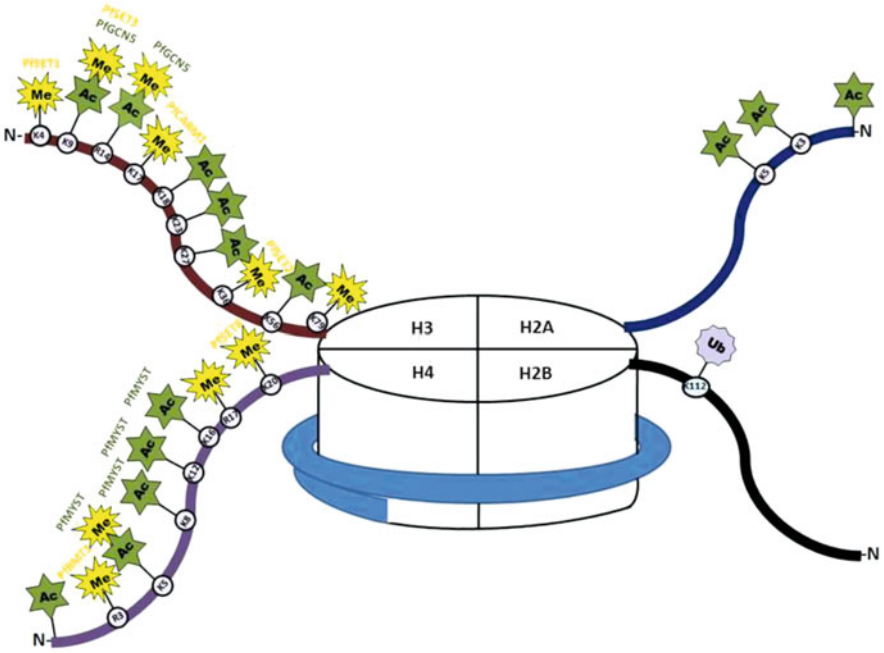

Fig. 28.1 The core histone (*H2A, H2B, H3* and *H4*) and the DNA wrapped with are shown. The lysine residues (*K*) along with their positions are marked. Different types of histone modifications are marked as followed: *Ac* acetylation, *Me* methylation, *Ub* ubiquitination. Several histone code readers like PfSET1, PfSET3, PfMYST etc. are shown (adopted from Cui and Miao 2010 and subsequently modified)

28.3.5 Histone Poly-ADP-Ribosylation

Poly-ADP ribose polymerase homologue has not been identified in *Plasmodium* indicating that histone Poly-ADP-ribosylation may be absent in the malaria parasites.

The nucleosome structure containing the core histones with different types of modifications are shown in Fig. 28.1.

28.4 Histone Code Readers

The arrays of histone tail modification are recognized by a number of conserved protein domains that facilitate downstream events via the recruitment or stabilization of chromatin-related protein complexes. These protein modules are classified into several subgroups like the bromodomain, Royal superfamily, plant homeodomain (PHD) fingers, WD40 repeats and 14-3-3 proteins (Deitsch et al. 2009; Taverna et al. 2007). Bromodomain is an evolutionary conserved acetyl lysine- binding module

found in many chromatin-associated proteins (Zeng and Zhou 2002). PHD fingers and the Royal superfamily protein fold, including chromodomain are methyllysine-binding modules (Maurer-Stroh et al. 2003). The WD40 repeat protein WDR5, a component of SET1 complex, binds unmodified H3R2 (Ruthenburg et al. 2006). The 14-3-3 proteins contain phosphoserine-binding modules which bind to H3 phosphoserine with high affinity and are involved in various cellular functions (Macdonald et al. 2005).

The malaria parasites contain a number of proteins with PTM-binding modules. The PfGCN5 HAT and PfSET1 HKMT proteins with a single chromodomain have been identified in *P. falciparum*. *P. falciparum* also contains a number of Royal superfamily and PHD finger domain containing proteins. The PfMYST and heterochromatin protein 1 (PfHP1) contain a single chromodomain. The PfHP1 protein containing a chromodomain and a chromo-shadow domain are involved in H3K9me3 binding and dimerization respectively (Flueck et al. 2009; Perez-Toledo et al. 2009). This protein is associated with the H3K9me3 marks in subtelomeric and intrachromosomal silent *var* genes correlating with silencing status of the *var* gene family. The dimerization is probably responsible for subtelomeric heterochromatin formation by aggregating nucleosomes in subtelomeric region. Ten PHD domain containing proteins, 90 WD40 motif containing proteins and three putative 14-3-3 motif containing proteins have been identified in *P. falciparum* so far. However, these proteins need to be functionally characterized. The details of the histone modifying enzymes and histone code readers are shown in Table 28.2.

28.5 Chromatin Remodelling Complexes

Transcriptional activation and silencing is brought about not only through array of histone post-transcriptional modifications but also through direct activity of chromatin remodelling complexes on nucleosomes. In eukaryotes, four different classes of ATP-dependent chromatin remodelling complexes (Swi/Snf, INO80, ISWI and Mi2/NURD) have been described (Martens and Winston 2003). The functions of Swi2/Snf2 family chromatin remodelers in protozoan parasites are not known clearly. In trypanosomes, a protein related to Swi2/Snf2 is involved in *de novo* synthesis of the modified thymine base 'J' within telomeric DNA, which correlates with the epigenetic silencing of variant surface glycoproteins (VSGs) (DiPaolo et al. 2005). Analysis of the *P. falciparum* genome identifies 11 Swi2/Snf2 type ATPases, characterized by an interrupted P-loop ATPase domain (Horrocks et al. 2009; Templeton et al. 2004). Swi2/Snf2 ATPase are major components of the SNF2 mediated DNA repair mechanism and four of these predicted Swi2/Snf2 ATPases share similarity with components of the Rad54 and Rad5/16 pathways (PF08_0126, MAL13P1.216, PFL2440w and PFF0225w). In *P. falciparum*, *var* gene activation is associated with the alteration of local chromatin structure and reduced nucleosomal occupancy at the promoters (Duraisingh et al. 2005; Voss et al. 2007; Westenberger et al. 2009). Therefore, it is important to identify the role of Swi2/Snf2 ATPase in antigenic variation.

Table 28.2 List of histone modifying enzymes (A) and proteins containing histone PTM-binding modules (B)

A.	
Gene ID	PlasmoDB
Histone acetyl transferase (HAT)	PF08_0034
PfGCN5	PF11_0192
	PFL1345c
	PFD0795
	PFA0465c
	PF14_0350
Histone deacetylase (HDAC)	PF1260c
PfHDAC1	PF14_0690
	PF10_0078
PfSir2	PF13_0152
	PF14_0489
Histone methyl transferase (HKMT)	
PfSET1	PFF1440w
PfSET2	MAL13P1.122
PfSET3	PF08_0012
PfSET4	PFl0485c
PfSET5	PFL0690c
PfSET6	PF13_0293
PfSET7	PF11_0160
PfSET8	PFD0190w
PfSET9	PFE0400w
Histone demethylases	Mal8P1.111
PfmjC1	PFF0135w
PfmjC2	PFL0575w
Protein Arginine transferase (PRMT)	
PfRMT1	PF14_0242
PfRMT4/PfCARM1	PF08_0092
PfRMT5	PF13_0323
Swi2/Snf2 ATPases	PF08_0048
PfSRCAP1	PF11_0053
PfSNFL	PFF1185w
	PFB0730w
	PF10_0232
	MAL8P1.65
	PF13_0308

B.		
Binding module/PTM mark	PlasmoDB	Annotation/PTM mark
Royal superfamily/Kme chromodomain	PF1005c	PfHP1/H3K9me3
	PF11_0192	PfMYST
	PF11_0418	

(continued)

Table 28.2 (continued)

B.		
Binding module/PTM mark	PlasmoDB	Annotation/PTM mark
Bromodomain/Kac	Pf08_0034	PfGCN5
	PFF14440w	PfSET1
	PFA0510w	
	PFL0635c	
	PFL1645w	
	PF10_0328	
	PF14_0724	
Double chromodomain Tudor domain	PF10_0232	PfCHD1
	PF11_0374	PfTSN
	PFC1050w	PfSMN
PHD finger/Kme	PFF1440w	PfSET1
	MAL13P1.122	PfSET2
	MAL13P1.302	
	PFC0425w	
	PF10_0079	
	PF11_0429	
	PFL1011c	
	PF14_0315	
	PFL0575w	
WD40 repeat	PFA0520c	CAF-1
	PFD0455w	CAF-1
14-3-3 protins/S_{ph}	MAL8p1.69	
	MAL13P1.309	
	PF14_0220	

Adapted from Chung et al. (2009), Cui and Miao (2010) and subsequently modified
K lysine, *me* methylation, *ac* acetylation

28.6 Nuclear Architecture of *Plasmodium falciparum*

Nuclear architecture also plays a crucial role in epigenetic control of transcriptional regulation. Nucleus includes some specific organelles like nucleolus, nuclear speckles, chromatin territories etc. These organelles and the specialized structures may have fundamental role in expressing or silencing specific genes based on their specific location in the nucleus. Proteins present in the nucleus may be either associated with DNA or responsible for the maintenance of nuclear architecture like nuclear envelope proteins or nucleoplasmic proteins.

Nucleolus is the most important sub compartment of nucleus which performs crucial functions like ribosome biogenesis and storage of specific proteins. Like yeast, *Plasmodium falciparum* nucleus shows only one large nucleolus that occupies a large proportion of nucleus at one pole with a hat like or crescent shaped structure (Figueiredo et al. 2005). It harbours proteins like Sir2, ORC1 (origin recognition complex subunit 1), small nucleolar RNP protein NOP1 (fibrillarin), TERT

(telomerase reverse transcriptase) and other important proteins (Figueiredo et al. 2005). Although nucleolus contains histone deacetylase Sir2 protein that may be involved in rDNA transcription, its role in epigenetics mediated gene regulation remains to be explored further.

Previous reports suggest chromosome specific cluster formation of telomeric ends in *Plasmodium falciparum* and these clusters are organized at the nuclear periphery (Mancio-Silva et al. 2008, 2010; Freitas-Junior et al. 2005). Telomeric ends accumulate various proteins like Sir2, ORC1, HP1 leading to the heterochromatin formation. However, the protein bound DNA structure slowly reorganizes as the cell cycle progresses (Freitas-Junior et al. 2005). It has been suggested that the region beneath the nuclear envelop acts as a silencing zone and in order to relieve the silencing effect of a specific gene, it has to come out from this silencing zone (Freitas-Junior et al. 2005; Duraisingh et al. 2005; Ralph et al. 2005). The telomere associated virulence gene (*var*) families undergo such kind of modification events. Following transcription, the transcripts are transported into the cytoplasm through the nuclear pores. The silencing zone may be disrupted at the nuclear pore complex region. All the above phenomena are depicted in the schematic diagram as shown in Fig. 28.2.

It has been found that central core area of nucleus beneath the periphery is responsible for active transcription of genes as suggested by co-localization with H3K9Ac that marks the active transcription (Lopez-Rubio et al. 2007; Issar et al. 2008b). However, peripheral circle beneath the nuclear membrane is marked by H3K9me3 suggesting repressive status corresponding to the location of telomeric clusters containing silent *var* genes (Issar et al. 2008b). Any silenced *var* gene may be activated only if its promoter region acquires histone modifications like H3K9Ac and H3K4me3 in the ring stage, the earlier stage of cell cycle (Chaal et al. 2010).

28.7 Pathophysiology of Malaria

Malaria is an intravascular infection. The symptoms of disease appear only due to destruction of erythrocytes and circulation of parasite and erythrocyte specific materials in blood stream of vertebrate host. It used to be the belief that parasite specific toxins are released in the blood stream when schizonts are ruptured. Now, it is established that malaria parasites induce release of some cytokines. Symptomatic malaria is found to be associated with the release of interferon gamma, TNF (tumor necrosis factor alpha), interlukein-1 beta (IL-1 beta) and IL-6 (Grau et al. 1989, 1997). The release of cytokines in response to parasite specific antigens and other debris in the form of lipid or protein is crucial for pathogenesis. Pathogenesis also involves rosette formation and heavy accumulation of large clumps of infected erythrocytes in the capillaries of vital organs like brain, placenta, lungs, and kidneys resulting in multi- organ failure.

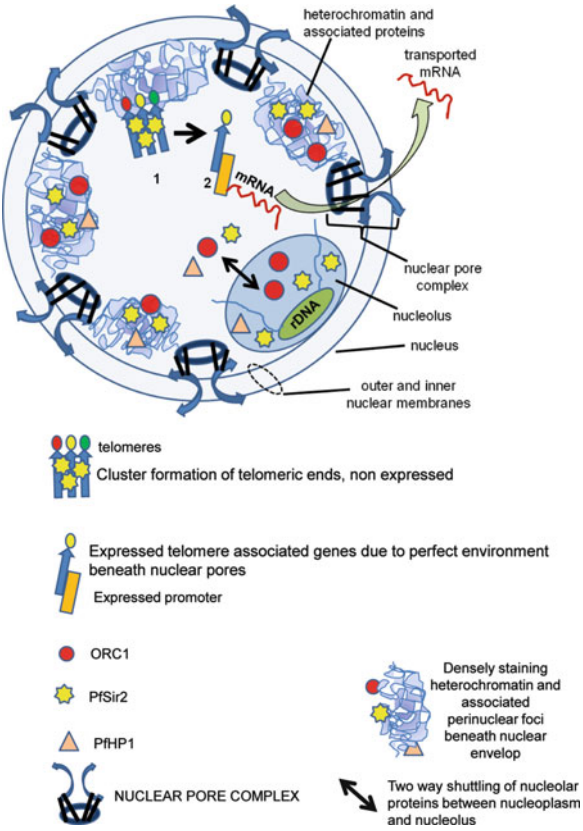

Fig. 28.2 Nuclear architecture of *Plasmodium falciparum*. The architecture of the nucleus with nuclear envelop, heterochromatin region containing clusters of telomeric region, nuclear pores, nucleolus are shown. Chromosomal ends with telomeric regions form clusters at the heterochromatin region containing several proteins like PfSir2, ORC1 involved in the silencing of *var* genes. Activation of *var* promoters require reorganization of the heterochromatin structure that will allow the specific gene to come out of this silencing zone. Nuclear pores may allow the transport of the transcripts following transcription. Nucleolus is involved in the transcription of rDNA that may require the presence of Sir2 and ORC1 in this organelle although their specific roles are not clear yet

28.7.1 Erythrocyte Rosetting

Erythrocyte rosetting is a phenomenon responsible for malarial parasite pathogenesis which involves complex formation of several red blood cells including both parasitized and uninfected cells. It includes arrangement of red blood cells around a central cell in a flower like manner. The surface of parasitized red blood cell shows the presence of *knob* like structures playing a key role in attachment of red blood cells leading to rosette formation (Aikawa et al. 1983; Gruenberg et al. 1983). Electron microscopy reveals the interaction of red blood cells at the *knobs*

(Scholander et al. 1998). Sequestration of parasitized red blood cells in vascular system is a characteristic event of *Plasmodium* pathogenesis. Sequestration is the removal of parasitized red blood cells from blood vascular system followed by specific interactions between vascular endothelial cells and parasite specific surface antigens which decorate the outer surface of red blood cell in a stage specific manner. Sequestration ultimately leads to vital organ failure. Literature shows the involvement of PfEMP1 in sequestration process responsible for escape from immune system. PfEMP1, a polypeptide of ~200 to ~350 kDa is the product of *var* gene family (Howard et al. 1983). PfEMP1 specifically recognizes host cell receptors CD36 and ICAM1 (Smith et al. 1995). PfEMP1 polypeptides are composed of an N-terminal Duffy binding like domain, DBL1, cysteine rich interdomain region (CIDR), a hydrophobic transmembrane region and an internal segment that is acidic in nature. Some of the severe effects of rosetting are described below.

28.7.1.1 Placental Malaria

Pregnant women are found to be highly susceptible to malaria. It has already been established that intense sequestration of parasitized red blood cells takes place in the placenta leading to the thickening of syncytiotrophoblasts (the multinucleated cells of placenta of embryos) and abnormal blood flow exchange between foetus and mother. Syncytiotrophoblast is the major foetal surface in contact with maternal blood. Production of inflammatory cytokines in response to heavy placental accumulation of parasitized red blood cells leads to the growth retardation of foetus and more than 50% women approaches death subsequently. No interaction takes place between uninfected erythrocytes and placental receptors showing the importance of parasite specific cell surface antigens involved in attachment with receptors. As already stated above, PfEMP1 molecules mediate interaction with placental surface receptors. (Salanti et al. 2003) has shown the involvement of a CSA binding parasite ligand var2CSA PfEMP1.

28.7.1.2 Cerebral Malaria

Plasmodium falciparum is the causative agent of cerebral malaria in humans and it is due to the accumulation of infected red blood cell clusters in cerebral blood capillaries followed by massive haemorrhage due to rupture of capillaries (Ho et al. 1991). Cerebral malaria is associated with increased cytokine secretion leading to TH-1 response. Tumor necrosis factor and some interleukins including IL-6, IL-8 etc. show elevated expression in response to parasite infection (Grau et al. 1997). Increased cytokines are responsible for symptoms of cerebral malaria like fever and shivering followed by coma and ultimately death. Rosette accumulation leads to symptoms of cerebral malaria and suggests the role of epigenetics control in *var* gene regulation.

28.7.2 Antigenic Variation

Antigenic variation is defined as a process in which an infectious organism changes its surface protein from one to another form thereby evading host immune response. Antigenic variation also enables the pathogens to reinvade the host immune system because the antigenic peptides are no longer recognized due to the high antigenic variability (Beeson and Brown 2002; Biggs et al. 1991). It is the characteristic event of many pathogenic organisms from viruses to protozoa like influenza virus, HIV, trypanosoma, neisseria, *Plasmodium falciparum* etc. Antigenic variation in *Plasmodium falciparum* involves different *var* gene families that are associated with subtelomeric regions. Regulation of these genes involves three dimensional repositioning of different *var* genes in the nucleus.

In general, antigenic variation can be in the form of (a) antigenic drift involving changes in a few amino acids of surface antigenic peptide or (b) antigenic shift showing acquisition of new surface proteins. *Plasmodium falciparum* shows antigenic drift of surface proteins encoded by *var* gene families. Influenza virus is the classical example of antigenic drift and antigenic shift types of variation involving hemagglutinin and neuraminidase. Antigenic variation in *P. falciparum* involves a multigene family of surface antigens. Literature suggests that every *var* gene consists of a 5′ exon followed by a small 3′ exon. First exon makes extracellular variable domain of PfEMP1 and small exon is specific for a cytoplasmic domain showing considerable homology among *var* gene repertoire. These multigene families are found to be associated at chromosomal ends in tandem repeats and ectopic recombination is the result of high antigenic diversity of *var* genes.

28.8 Epigenetic Control and *var* Gene Expression

Central to the pathogenesis of *Plasmodium* as described above is the variation in the expression of *var* gene encoded PfEMP1 protein responsible for cytoadherence and antigenic variation. As described above, only one *var* gene is expressed in an allele specific manner while 59 *var* genes remain silenced. The switching of *var* gene expression is the key to evade the host immune system. The link between the *var* gene expression and epigenetic control has been demonstrated nicely. It has been shown that the promoter region of a *var* gene when cloned in a transfection vector leads to constitutive expression rather than stage specific expression in the parasites (Deitsch et al. 1999; Voss et al. 2003).

The epigenetic control of *var* gene regulation was further strengthened when two independent groups showed that the deletion of histone deacetylase Sir2 homolog in *Plasmodium* (Prusty et al. 2008; Merrick and Duraisingh 2007) could result in the derepression of some but not all *var* genes (Duraisingh et al. 2005). Further, the association of PfSir2 with the telomeric region and telomeric associated repeat elements (TARE) was confirmed by chromatin immunoprecipitation (ChIP)

experiments and electrophoretic mobility shift assay (EMSA) (Mancio-Silva et al. 2008). Similar results have been reported for another protein involved in heterochromatin formation, *P. falciparum* heterochromatin protein 1 (PfHP1) (Flueck et al. 2009; Perez-Toledo et al. 2009). It is possible that PfSir2 generates a heterochromatin region by spreading from telomeric to TAREs thereby affecting the expression of the *var* genes adjacent to the TARE regions. Another protein that has been implicated in this process is PfORC1, a replication initiation protein also involved in *var* gene silencing possibly by facilitating the heterochromatin formation in coordination with Sir2. Preliminary results indicate that the N-terminal domain of PfORC1 is involved in the process in a Sir2 dependent manner. PfHP1, also implicated in the heterochromatin formation may not be involved directly in this process (Deshmukh et al. 2012).

It has been further demonstrated that the promoter of var2CSA, a specific *var* gene is occupied with Sir2 only when it is inactive. Additionally, the same promoter is occupied with acetylated histone H4 only when it is transcriptionally active and not inactive (Freitas-Junior et al. 2005). Taken together, it can be concluded that var2CSA is regulated by epigenetic control of the promoter by reversible histone acetylation and deacetylation.

A more detailed study was performed later to investigate the histone modification status of the promoter of var2CSA during different stages. It has been shown that the 5′UTR region of active var2CSA is enriched with acetylated H3K9 and di and tri- methylated H3K4 during the ring stage of the parasite development. However, the di-methylated form of H3K4 is predominant during the trophozoite and schizont stage of the parasite life cycle when var2CSA is not active (Lopez-rubio et al. 2007). It has also been shown that H3K9me3 is enriched at the 5′UTR region of var2CSA and throughout the coding region when it is inactive. Thus the trimethylated form of H3K9 and acetylated and trimethylated form of H3K4 are the epigenetic markers that dictates the repressed and active state of var2CSA respectively in *Plasmodium falciparum*. Although the epigenetic control of *var* gene regulation has been established, it is still not clear how a particular type of *var* gene is activated out of many such genes.

Recently epigenome mapping of *P. falciparum* revealed interesting results. The MS analysis of histone modifications revealed the existence of H3/H4 acetylation and H3K4me3. ChIP on chip profiling of different acetylated and methylated form from asynchronous parasites suggest the presence of surface antigen gene families in the heterochromatin region. However, analysis of synchronized parasites revealed unique results. In ring, both H3K4me3 and H3K9me mark both active and inactive genes equally. However, during schizont, only the 5′ upstream regions of the active genes are marked by them. These striking differences during ring and schizont stages clearly indicate that the epigenetic marking in the parasites is a dynamic process that undergoes several changes throughout the erythrocytic stages (Flueck et al. 2009). These results also suggest that epigenetic control in the parasites are far more complex that need to be evaluated thoroughly in order to correlate the transcriptome map and the epigenome map at a very high resolution at the different erythrocytic developmental stages.

28.9 HDAC and HAT Inhibitors for Therapeutics

Histone epigenetic code is a key regulator of eukaryotic gene expression. In eukaryotes, histone deacetylase (HDAC) and histone acetyltransferase (HAT) enzymes control the acetylation of histones (lysine residues within histone tails) and nonhistone proteins and thus are important for various cellular functions, such as transcription, DNA replication and repair, cell signalling, cell cycle regulation and differentiation (Xu et al. 2007; Yang and Seto 2007). Equilibrium between HAT and HDAC activities must be maintained for proper transcriptional activity and above-mentioned cellular functions. Therefore, deregulation of HDAC activity has been recognized as an important therapeutic aspect used in cancer cells. Supberoylanilide hydroxamic acid (SAHA) (Vorinostat) inhibitor of HDAC is approved for the treatment of T cell lymphoma (Grant et al. 2007).

Widespread multi-drug resistance to malaria and ineffective vaccines have made global efforts to eradicate malaria very difficult. Therefore, there is urgent need to develop new classes of antimalarial compounds to combat this disease. Malaria parasite undergoes significant morphological changes during its asexual life cycle in humans, during transmission from the insect vector to the human host. Therefore, appropriate control of histone acetylation is important for parasite survival. The HDACs have been evaluated as promising drug targets, and many HDAC inhibitors possess potent antimalarial activities (Andrews et al. 2009). The HDAC inhibitor elicits an increase in *P. falciparum* histone acetylation with reduced parasite proliferation, which suggests importance of HDAC function (Chaal et al. 2010). The HDACs have been identified as important regulators of transcription in *P. falciparum*. Apicidin, a cyclic tetrapeptide isolated from Fusarium spp., affects both class I and II HDACs in the parasite and it causes profound transcription changes within 1 h of treatment leading to the cell cycle arrest of the parasites (Darkin-Rattray et al. 1996; Chaal et al. 2010). Apicidin also induces expression of stage specific genes that are otherwise suppressed during that particular stage of intraerythrocytic developmental cycle (IDC) in *P. falciparum*. Nicotinamide, an inhibitor of PfSir2A has been reported to affect parasite growth at high concentration (Prusty et al. 2008). Other inhibitors such as SAHA, trichostatin and hydroxamate derivatives have also been shown to exhibit antimalarial effects (Jutta et al. 2011; Andrews et al. 2008; Colletti et al. 2001; Dow et al. 2008).

Histone acetylation in the parasite has been shown to regulate the monoallelic expression of the *var* genes, which mediates the antigenic switching and virulence of the parasite (Duraisingh et al. 2005; Freitas-Junior et al. 2005; Voss et al. 2006, 2007). Variegated expression of genes essential for erythrocyte invasion in different parasite clones are under epigenetic control suggesting conserved epigenetic mechanism for transcriptional regulation in malaria parasites (Cortes et al. 2007). The H3 acetylation by GCN5 plays important role in the parasite gene activation and inactivation of histone acetylation compromises the parasite development suggesting its role for viable drug targets (Cui et al. 2007; Fan et al. 2004a, b). The downregulation and inhibition of PfGCN5 activity by curcumin and anacardic leads

to parasite growth inhibition (Cui et al. 2007, 2008a, b). Treatment with Anacardic acid for 12 h induced twofold or greater changes in the expression of ~5% of genes in *P. falciparum* trophozoites, among which 76% were downregulated (Cui et al. 2008a, b). Therefore, the effect of these inhibitors in histone hypoacetylation and downregulation of developmentally regulated genes in the parasite may have great potential for parasite survival and growth.

28.10 Concluding Remarks

Revolutionising advances in the study of epigenetic control of gene expression did not leave apicomplexan *P. falciparum* unaffected especially following characterization of mutually exclusive expression of *var* gene family in 1998. The studies gained momentum in post genomic era when DNA sequences were available for scrutiny. The parasite displays considerable conservation of epigenetic mechanisms in having a typical nucleosome organization, numerous histone posttranslational modifications, an array of conserved chromatin modifications and remodelers with histone binding modules. However, there are some divergences like canonical eukaryotic histone code H3K4me2 that appears to provide a novel heritable mark for expression of *var* gene in the subsequent generations and lack of distinct PTMs like H3K27me, H3K79me in *Plasmodium*. These differences suggest a unique parasite specific epigenetic mechanism with its novel set of histone code writers and their cognate readers.

Progress of epigenetic studies in the parasite is helping to understand the overall pathophysiology of the parasite including the critically important antigenic variation as well as erythrocyte invasion pathways. However, our understanding of the *Plasmodium* epigenetics is marred by several limitations. This includes lifecycle limitations where the studies have been predominantly limited to the erythrocytic stages that too in long term cultured laboratory strains with *in vitro* conditions that are not representative of *in vivo* situations. The significance of erythrocytic stage can never be underestimated considering that it is the stage with disease manifestations and pathogenesis but other stages of the complicated life cycle need attention as it is known to have distinct transcriptional profile. Practical limitations of parasite material in hepatocyte culture and mosquito stages have become a hindrance in studying these stages. Also, the effect of host immune system on the epigenetic regulation of the parasite especially in the context of antigenic variation needs to be probed. Epigenetic studies in the *Plasmodial* parasite are still nascent but will surely further the understanding of the uniquely complex lifecycle of this parasite and in the process may offer some valuable targets for anti-malarial chemotherapeutics.

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