

HETEROCHROMATIN EFFECTS IN FRIEDREICH'S ATAXIA AND SEXUAL DIMORPHISM

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for the degree of Doctor of Philosophy

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London, April 2012

Declaration of originality

Hereby I confirm that this thesis is a result of my own work and findings or ideas by others are appropriately referenced throughout the text. Collaborations are mentioned at the end of each results chapter.

Abstract

Heterochromatin is implicated in the negative regulation of gene expression. To understand the effects of heterochromatin on RNA polymerase-II (RNAPII) mediated transcription, this study focused on the *FXN* gene where abnormal silencing induced by expanded (GAA)_n repeats causes Friedreich's ataxia (FRDA), an incurable neurological disorder. Here, the silenced *FXN* locus was found to be modified by the heterochromatic histone marks H3K9me3, H3K27me3 and bound by HP1 β . This pathological heterochromatinisation was partially reversed by the histone deacetylase inhibitor nicotinamide, which upregulated *FXN* expression to potentially therapeutic levels *in vitro*, *ex vivo* and *in vivo*. In addition, RNAPII was shown to be stalled in the first exon of the *FXN* gene and degraded by the proteasome in FRDA but not in healthy cells. This was linked to increased proteasome binding at the silenced *FXN* locus in a pattern reminiscent of the heterochromatin marks. Importantly, proteasome inhibition restored stalled RNAPII levels in FRDA and upregulated *FXN* to potentially therapeutic levels *in vitro*. Moreover, experiments with healthy human cells and wild-type mouse thymus revealed enriched levels of proteasome binding in other heterochromatic regions (e.g. pericentromeric repeats and SINEs) which were also de-repressed by proteasome inhibition; suggesting that the effect seen on the pathological *FXN* locus represented a specific example of a more generalised phenomenon. Overall, this introduces a novel mechanism whereby heterochromatin might be maintained in a silent state.

In this thesis, heterochromatin effects were also investigated in relation to sexually dimorphic gene expression. Microarray analyses revealed hundreds of autosomal genes sensitive to sex chromosome-complement rather than gender. HP1 β -repressed genes and SINE elements were over-represented within this gene group suggesting a potential link between heterochromatin, proteasome-dependent silencing and sexually dimorphic gene expression. The results reveal a novel layer in the regulation of sexually dimorphic genes with implications for understanding sex-bias in physiology and disease.

Publication

Wijchers, P.J., **Yandim, C.**, Panousopoulou, E., Ahmad, M., Harker, N., Saveliev, A., Burgoyne, P.S., and Festenstein, R. (2010). Sexual dimorphism in mammalian autosomal gene regulation is determined not only by Sry but by sex chromosome complement as well. *Dev Cell* 19, 477-484.

Dedication

...To my beloved family;

my mother Ayten and father Ahmet.

Also to my lovely grandparents, aunts and dearest cousin;
my life-long friends Derya and Sezgin;
and best friends in the United Kingdom: Emine, Pelin, Gamze.

Acknowledgements

This Ph.D. study has been the most challenging part of my life so far. Coming to the end of this long marathon, I think I have learned invaluable lessons not only in science but also in many other aspects of life. This would not be possible at all if it wasn't for my dear supervisor Richard Festenstein, who kindly accepted me as a Ph.D. student and supported me all the way through. Therefore, I also think I am very lucky...Thank you Richard, for being a really supportive and empathetic supervisor.

Very special thanks to my friendly colleagues Raul Torres and Jackson Chan, who always shared their experiences and bright ideas with me. The projects presented here would never have reached this stage without their generous help. I would also like to thank Maria Mayan for teaching me the ChIP technique and for her collaboration in the Friedreich's ataxia project. Similarly, Patrick Wijchers provided incredibly good supervision in the sexual dimorphism project and he deserves a big thank you... Anne Bygrave and Andrew Georgiou were both like helping angels in the laboratory with years of scientific experience. Dear Anne, thank you for being a good and trustworthy friend. Emily Ferencz, I will never forget your kind manners and great help in the early days of this Ph.D. Theona Natisvili, you were always a positive and hard-working student. I always felt privileged to teaching you in the laboratory. Ecem Sener, you are one of the brightest students I have ever seen. Thank you so much for your help in the laboratory and more importantly for your sincere friendship. I will always remember the summer in London in 2010. Pui-Pik Law, I always felt happy and will feel happy to collaborate with a hard-working and friendly colleague like you. Nora Anghelescu, you cheered me up every time I saw you and became a good friend. Thank you for being on my side at difficult times. Lastly, but not least, I managed to meet a distinctive Japanese scientist...Kyoko, thank you for your support during the stressful days at the end of this Ph.D. All the other members of GCMD from past to now...Benoit, Phil, Vineet, Sophia, Natasha and many others ...Thank you all for your help and friendliness. I should also express my gratitude to my advisors Jesus Gil and Ana Pombo for their kind helps. Likewise, I am thankful to our senior post-graduate administrator Anne Soutar as well as Geoffrey Kembell-Cook and Kate Baird, who continuously and willingly helped students at the MRC CSC. I also acknowledge Mark Pook and other members of EFACTS for useful communication and sharing materials. Special thanks to my examiners for taking time to read this manuscript.

Finally, I am thankful to the Medical Research Council of United Kingdom for creating this Ph.D. opportunity and generously funding this studentship.

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* 'S' stands for supplementary

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* 'S' stands for supplementary

Abbreviations

%	percent
%v/v	volume/volume
%w/v	weight/volume
°C	degree Celsius
3'	three prime end
3C	chromosome conformation capture
4C	circular chromosome conformation capture
5'	five prime end
A	adenine
a-am	alpha-amanitin
ac	acetylated
ADA	P300/CBP-associated factor
AKT1	v-akt murine thymoma viral oncogene homologue 1
ALDOA	aldolase A
AMP	adenosine monophosphate
AMPK	AMP activated protein kinase
AR	androgen receptor
ART	ADP ribose transferase
ASH1	absent, small, homeotic discs related protein (methyltransferase)
ATM	<i>Ataxia Telangiectasia</i> mutated
ATP	adenosine 5'-triphosphate
BAX	Bcl-2-associated X protein
BER	base excision repair
BML-210	<i>N</i> ¹ -[2-aminophenyl]- <i>N</i> ⁸ -phenyloctanediamide
bp	base pair
BRCA	breast cancer associated protein
BRE	TFIIB response element
BSA	bovine serum albumin
C	cytosine
CAF1	chromatin assembly factor
CBP	CREB binding protein
CBX	chromobox protein
CDK	cyclin dependant kinase
cDNA	complementary deoxyribonucleic acid
CE	capping enzyme
CENP	centromere protein
ChIP	chromatin immunoprecipitation
CoA	coenzyme A
CoREST	co-repressor of REST
CpG	cytosine-phospho-guanine
CREB	c-AMP response element binding protein
CSB	cockayne syndrome B
CSC	Clinical Sciences Centre
CSD	chromo-shadow domain
CSTF	cleavage stimulatory factor
CTCF	CCCTC-binding factor

CTD	C-terminal domain
CTIP	B-cell CLL/lymphoma 11B (zinc finger protein)
DAPI	4',6-diamidino-2-phenylindole
dH₂O	distilled water
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
DNMT	deoxyribonucleic acid methyl transferase
dNTP	deoxyribonucleotide triphosphate
DOT1	disruptor of telomeric silencing (methyltransferase)
DPE	downstream promoter element
DRB	5,6-dichloro-1 β -ribofuranosylbenzimidazol
DSB	double strand break
DSIF	DRB sensitivity inducing factor
dsRNA	double stranded RNA
DUB	deubiquitinating enzyme
<i>E. coli</i>	<i>Escherichia coli</i>
e. g.	for example
E1	Ubiquitin-activating enzyme
E2	Ubiquitin-conjugating enzyme
E3	Ubiquitin ligase
EBV	Epstein Barr virus
EC	elongation complex
EDTA	ethylenediaminetetraacetic acid
EEC	early elongation complex
ELL	eleven-nineteen lysine rich leukemia
ER	estrogen receptor
ES	embryonic stem cell
<i>et al.</i>	et alii
EtOH	ethanol
Ex1	exon 1
FACS	fluorescence activated cell sorting
FACT	facilitates transcription complex
FAD	flavine adenine dinucleotide
FCG	four core genotype
FCP1	F-cell production 1 (phosphatase)
FCS	fetal calf serum
Fe	Iron
FISH	fluorescence in situ hybridisation
FITC	fluorescein isothiocyanate
<i>FMR1</i>	Fragile X mental retardation 1 gene
FOXO	forkhead box proteins group O
FRDA	Friedreich's ataxia
<i>FXN</i>	human frataxin locus
Fxn	mouse frataxin locus
FXN	FXN protein

G	guanine
GATA	GATA binding transcription factor
GFP	green fluorescent protein
GH	growth hormone
GNAT	Gcn5-related N-acetyltransferase
GO	gene ontology
GTF	general transcription factor
H	histone
H₂O	water (molecule)
H3ac	histone H3 acetylation
H3K27me3	histone H3 lysine 27 tri-methylation
H3K9me3	histone H3 lysine 9 tri-methylation
H4ac	histone H4 acetylation
HAT	histone acetyl transferase
HD	Huntington's disease
HDA1	histone deacetylase 1
HDAC	histone deacetylase
HDACi	histone deacetylase inhibitor
HECT	Homologous to the E6-AP Carboxyl Terminus domain
HIF	hypoxia inducible factor
HIRA	histone regulator A
HIV	human immunodeficiency virus
HKDM	histone lysine demethylase
HKMT	histone lysine methyltransferase
HMBS	hydroxymethylbilane synthase
HMG	high mobility group
HOX	homeotic box group of proteins
HP	haptoglobin
HP1	heterochromatin protein 1
HPRT	hypoxanthine phosphoribosyltransferase
HR	homologous recombination
HRP	horseradish peroxidase
Hs	<i>Homo sapiens</i>
HSP	heat shock protein
HSS	hypersensitive site
HSV	Herpes simplex virus
i.e.	that is
IC₅₀	half of the maximum inhibitory dose
IF	immunofluorescence
Inr	initiator
Int1	Intron 1
iPS	induced pluripotent stem cell
ISC	iron sulfur cluster
JARID	jumonji AT-rich interactive domain protein (demethylase)
JHDM	jumonji domain containing histone demethylase
Jmj	Jumonji domain

JMJD	jumonji domain containing demethylase
K	lysine
KAP2	keratin associated protein 2
kb	kilobase pairs
KEGG	Kyoto Encyclopedia of Genes and Genomes
kg	kilogram
KI	knocked in
KO	knocked out
KRAB	Kruppel-associated box
l	litre
LCR	locus control region
LD₅₀	half of the lethal dose
LINE	long interspersed element
LSD1	lysine specific demethylase 1
LTR	long terminal repeat
M	molar
MAR	matrix attachment region
me	methylated
MeCP	methylated CpG binding protein
MEF	mouse embryonic fibroblast
MEF2	myocyte enhancer factor 2
MER	mariner DNA transposon
Mg	magnesium
mg	milligram
MG132	(Z-Leu-Leu-Leu-al) peptide aldehyde proteasome inhibitor
min	minutes
MIR	mammalian interspersed repeats
ml	millilitre
MLL	mixed lineage leukemia factor
mM	millimolar
Mm	Mus musculus
MMR	mismatch repair
MOF	<i>Drosophila</i> males absent on the first (acetyltransferase)
MOG	myelin oligodendrocyte glycoprotein
MOZ	monocytic leukemia zinc finger
MRC	Medical Research Council
mRNA	messenger RNA
MSH	mut S homologue (<i>E. coli</i>)
MSY	male specific region Y
MTE	motif ten element
MYSY	MOZ-YBF2-SAS2p-TIP family
NAD⁺	Nicotinamide adenine dinucleotide
Nam	Nicotinamide
NCBI	National Centre for Biotechnology Information
N-CoR	nuclear receptor co-repressor
ncRNA	non-coding RNA

NEDD4	neural precursor cell expressed, developmentally down-regulated 4 (ubiquitin ligase)
NELF	negative elongation factor
NER	nucleotide excision repair
ng	nanogram
NHEJ	non-homologous end joining repair
NIH	National Institute of Health
NIMR	National Institute of Medical Research
NMN	nicotinamide mononucleotide
NMNAT	nicotinamide mononucleotide adenylyltransferase
nt	nucleotide
NuRD	nuclear remodeling deacetylase
NURF	nucleosome remodeling factor
ORC	origin recognition complex
P16	Tumour suppressor protein P16
P21	tumour suppressor protein P21
P53	Tumour suppressor protein P53
P53BP2	P53 binding protein 2
PAAF1	proteasome ATP-ase associated factor
PAR	pseudoautosomal region
PARP	poly-ADP-ribose polymerase
PAX	paired box (group of proteins)
PBEF	pre-B-cell enhancing factor
PBS	phosphate buffered saline
PCF11	cleavage and polyadenylation factor subunit, homologue
PCR	polymerase chain reaction
PEV	position effect variegation
pH	pH value
PIC	preinitiation complex
PMSF	phenylmethanesulfonylfluoride
PPAR	peroxisome proliferator activated receptor
PRC	polycomb repressive complex
PRDM2	PR domain containing 2 (methyltransferase)
PRE	polycomb response element
PS341	(Pys-Phe-boro-Leu) boron modified peptide aldehyde proteasome inhibitor
P-TEFb	positive transcription elongation factor b
Q-RT-PCR	quantitative real time PCR
R	purine
RAD21	double-strand-break repair protein, yeast rad21 homologue
RASSF1	Ras association domain family member 1
RB	retinoblastoma protein
rDNA	ribosomal RNA coding DNA
RDP3	yeast reduced potassium dependency protein (deacetylase)
RDRP	RNA dependent RNA polymerase
RING	really interesting new gene protein(zinc finger protein)
RIP	RNA immunoprecipitation
RISC	RNA induced silencing complex

RITS	RNA-induced transcriptional gene silencing complex
RNA	ribonucleic acid
RNAi	RNA interference
RNAPII	RNA polymerase II
RNase	ribonuclease
ROS	Reactive oxidative species
RPA	replication protein A
RPB	RNA polymerase B (II) subunit
rpm	rounds per minute
RPN	regulatory particle non-ATPase (proteasome subunit)
RPT	regulatory particle triple (proteasome subunit, ATPase)
RUNX2	Runt domain transcription factor
<i>S pombe</i>	<i>Saccharomyces pombe</i>
<i>S. cerevisiae</i>	<i>Saccharomyces cerevisiae</i>
S2p	phosphorylated at serine 2
S5p	phosphorylated at serine 5
SAF1	scaffold attachment factor 1
SAGA	Spt-Ada-Gcn5-Acetyltransferase
SAHA	suberoylanilide hydroxamic acid
SAM	S-adenosyl-L-methionine
SAR	scaffold attachment region
Sat	satellite DNA
SCA	spinocerebellar ataxia
SCC1	sister chromatid cohesion protein 1
SCP1	small CTD phosphatase 1
SCS	Sex chromosome complement-sensitive
SDS	sodiumdodecylsulfate
SEM	standard error of the mean
SEN1	Senataxin 1
Ser	serine
SETDB1	SET domain bifurcated 1 (methyltransferase)
SF1	steroidogenic factor 1
shRNA	small hairpin RNA
SIN3	switch independent 3
SINE	short interspersed element
SIR2	silent information regulator 2
siRNA	small interfering RNA
SIRT1	sirtuin 1
SL(X/Y)	SYCP3-like X/Y-linked gene
SMC3	structural maintenance of chromosomes 3 (cohesin subunit)
SMRT	silencing mediator of retinoic acid and thyroid hormone receptor
SMYD3	SET and MYND domain containing 3 (methyltransferase)
SNP	single nucleotide polymorphism
SOX	SRY like box
SP1	specificity protein (transcription factor)
SRC1	steroid receptor co-activator 1

SRF	serum response factor
SRY	male sex determining region of Y
SSU72	Suppressor of SU7, gene 2 (yeast) homologue (phosphatase)
SUV39H	<i>Drosophila</i> supressor of variegation 3-9 (human homologue)
T	thymine
TATA	TATA box (DNA)
TBP	TATA binding protein
TCR	transcription coupled repair
TEMED	N,N,N,'N-tetramethylethylenediamine
TF	transcription factor
TIF	transcription intermediary factor
TiGER	tissue specific gene expression database
TIP	Tat interacting protein
TRAP	tagging and recovery of associated proteins
TRAPP	transcriptional adaptor protein
Tris	Tris (hydroxymethyl)aminomethane
tRNA	transfer RNA
TRX	trithorax proteins
TSA	trichostatin A
TSS	transcription start site
ub	ubiquitinylated
USF	upstream transcription factor
UT(X/Y)	ubiquitiously expressed X or Y (demethylase)
UTR	untranslated region
UV	ultra violet
Vol	volume(s)
W	A or G
Wt	wild type
XCI	X chromosome inactivation
Xi	inactivated X chromosome
XPG	<i>Xeroderma pigmentosum</i> G (exonuclease)
XRN2	5'-3' exoribonuclease 2
Y	pyrimidine
Zn	zinc

CHAPTER 1
GENERAL INTRODUCTION

1.1 Overview

There are approximately 25,000 genes in the human genome which are organised into 23 chromosome pairs in a landscape where they are interrupted with intergenic sequences and repetitive DNA. All somatic cells possess this same genetic information, which gives rise to more than 200 different cell types in the human body. This is only possible through highly orchestrated gene control mechanisms. Any dysregulation in this control system results in numerous diseases including cancer or neurodegenerative, immunological and many other pathological conditions. Therefore, one needs to understand the key events in gene regulation in order to develop rational medical strategies.

The central dogma of molecular biology (DNA→RNA→protein) dictates that the genetic code is transcribed into RNA which is subsequently translated to proteins. Interestingly, each step in this central dogma is subject to tight regulation. In this Ph.D. study, the main focus is the chromatin mediated regulation of transcription. In order for protein coding genes to be expressed, RNA polymerase II (RNAPII) and its components have to contend with relatively inaccessible forms of chromatin (i.e. heterochromatin). Accessibility of chromatin is mediated by a multitude of proteins with which DNA interacts. Such interactions are frequently regulated by post-translational modifications of these proteins as well as direct methylation of DNA or RNA interference based mechanisms. Moreover, DNA content (i.e. repetitive DNA) plays a significant role in determining the extent of chromatin compaction. Importantly, these regulatory events or their consequences can be inherited independently of the genetic code; a phenomenon known as “epigenetics”.

Most of these factors involved in the regulation of gene expression at different levels will be outlined in this chapter.

1.2 Chromatin structure

1.2.1 Hierarchical packaging of DNA

The stretched length of DNA in a eukaryotic cell is approximately 2 m long. The packaging of this large molecule containing the genetic code into a tiny cell nucleus, can only be possible by its hierarchical compaction (textbook-Alberts *et al.*, 2008). Structural packaging of DNA has been extensively studied mainly via microscopic studies and accessibility assays which are based on digesting genomic DNA with nucleases. As illustrated in figure 1.1, it all begins with the wrapping of a 145-260 bp stretch of DNA around a protein complex known as “nucleosome” (Kornberg, 1974). Nucleosomes are joined via an 8-114 bp long ‘linker’ DNA, forming a 10 nm thick ‘beads-on-a-string’ conformation. The second level of folding occurs when a series of nucleosomes are coiled around each other constituting a helical array known as the “30 nm chromatin fibre” (Finch and Klug, 1976). This self-folding hierarchy continues until the DNA is fully packed into 700 nm thick chromosomes, which are only visible during metaphase of cell cycle. During interphase; however, chromatin has lower levels of compactness ranging from 10-100 nm and the degree of folding is not uniform (Kornberg, 1977; Woodcock and Ghosh, 2010) . It is noteworthy that *in vivo* existence of this generally accepted hierarchical organisation has recently been questioned (Eltsov *et al.*, 2008; Robinson *et al.*, 2006).

Intra-nucleosomal interactions as well as ionic conditions have been associated with the array-like structure of chromatin (review-Woodcock and Ghosh, 2010). Moreover, scaffold (SAR) or matrix attachment regions (MAR) are anticipated to create anchor points in order to establish a ‘karyoskeleton’ inside the nucleus (Mirkovitch *et al.*, 1984). Neither the sequences of these elements nor the proteins involved for this model have been sufficiently clarified yet. There are also many other chromatin related proteins involved in chromatin compaction. These are frequently linked to transcriptional silencing and will be discussed in the following sections.

Figure 1.1

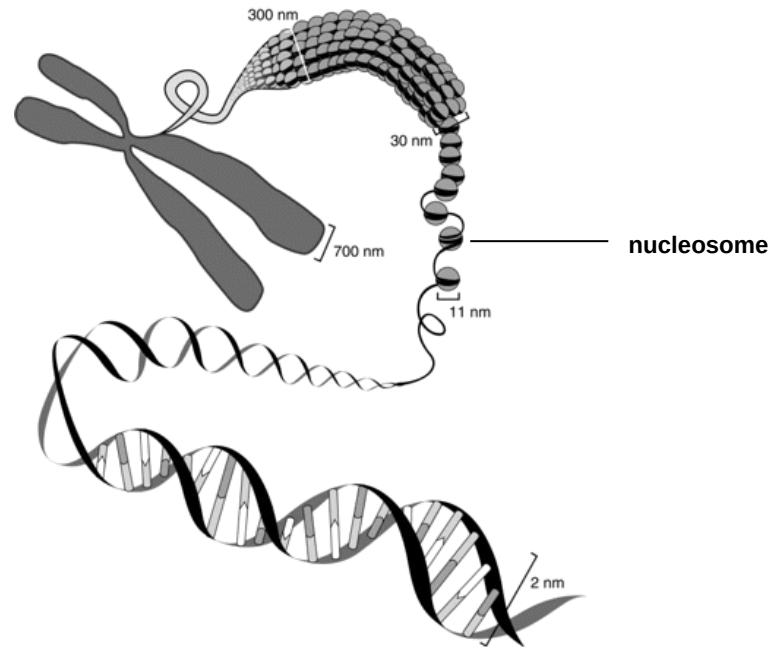


Figure 1.1 - Packaging of DNA into nucleus. It all starts with the wrapping of DNA around nucleosomes forming a 'beads-on-a-string' model. Coiling of nucleosomes around each other forms a '30 nm chromatin fibre'. This self-folding hierarchy continues until DNA is packed into metaphase chromosomes. [Adapted from (review-Weier, 2001)]

1.2.2 Euchromatin and heterochromatin

When examined under the microscope after DNA staining, an interphase cell nucleus typically looks like a mosaic image as a result of densely and lightly stained regions of chromatin [Figure 1.2]. Lighter regions are known as “euchromatin” and darkly stained compartments refer to “heterochromatin” (Heitz, 1928). Euchromatin, which is rich in transcriptionally active genes, is thought to be easily accessible to transcriptional activators and RNA Polymerase II (RNAPII), but less accessible and highly compact heterochromatin is associated with transcriptional repression as well as centromeric and telomeric repeats. Other characteristics of heterochromatin are that it is more prevalent in non-dividing and differentiated cells, it forms clusters predominantly close to the nuclear periphery, replicates late in S-phase and shows reduced recombination rates (review-Dillon, 2004). For an interphase cell, it is thought that transcriptionally active euchromatic genes usually have a ‘beads-on-a-string’ conformation of chromatin, whereas inactive heterochromatic genes are thought to be packed into a ‘30 nm chromatin fibre’ (Ghirlando and Felsenfeld, 2008; review-McKeown and Shaw, 2009).

Heterochromatin can be further sub-classified into ‘constitutive’ and ‘facultative’ forms (Natarajan and Schmid, 1971). Constitutive heterochromatin is enriched in non-coding repetitive elements such as centromeric, satellite or telomeric DNA. On the other hand, facultative heterochromatin is an intermediate form and can be established through the silencing of formerly euchromatic genes. A good example for facultative heterochromatin is the inactivated X chromosome in females, which can be distinguished as a ‘Barr body’ by microscopy (review-Chadwick and Willard, 2003a; review-Dillon, 2004).

Figure 1.2

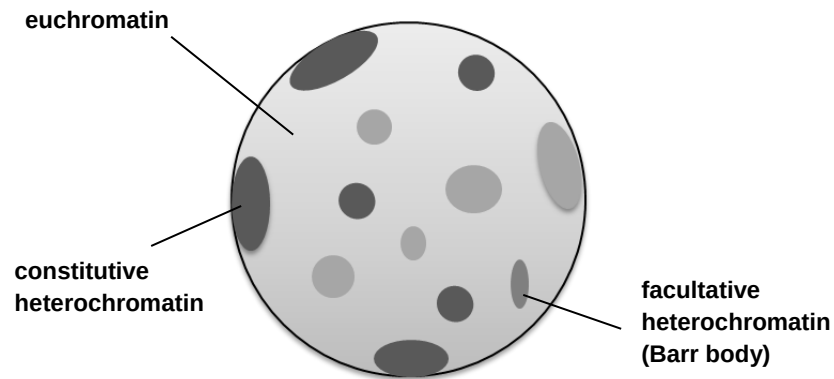


Figure 1.2 - Different compartments of chromatin in an interphase nucleus. Dense DNA staining represents the compact state of chromatin (referred as “heterochromatin”), whereas lightly stained regions comprise open state chromatin (referred as “euchromatin”).

1.2.3 Nucleosome

In order to understand gene regulation at the chromatin level, one needs to have a closer look at its fundamental subunit, the 'nucleosome'; because it is here that much of the variation begins. A nucleosome is composed of highly conserved small proteins called "histones (H)". Nucleosome assembly takes place with the deposition of an H3/H4 tetramer on the DNA, followed by two sets of H2A/H2B dimers [Figure 1.3A] (Luger *et al.*, 1997). 'Linker' histone H1 is found outside the core complex, mainly interacting with the linker DNA, and triggers chromatin compaction [Figure 1.3B] (Allan *et al.*, 1980). Molecular interactions between linker histones on one hand, and interactions between intra-nucleosomal H2A and H4 on the other, are thought to play an essential role in the maintenance of chromatin fibres (Dorigo *et al.*, 2004; Thoma and Koller, 1977).

In terms of structure, histones are generally composed of a globular domain and a flexible N-terminal 'histone tail', which protrudes from the surface of nucleosome. All four core histones possess a helix-turn-helix-turn-helix motif, which allows their dimerisation. The positive charge of the highly basic histones plays a key role in their interaction with the negatively charged DNA. Some nonpolar interactions between the backbone and deoxyribose sugar of DNA are also thought to facilitate this interaction (Luger *et al.*, 1997). Linker histones are thought to mainly interact electrostatically with the major groove of DNA via their globular winged-helix motif (Cerf *et al.*, 1993). Histones are mainly synthesised during the S phase of the cell cycle and replace the nucleosomal gaps that would otherwise result following DNA replication. This nucleosome assembly is facilitated by histone chaperones, mainly via chromatin assembly factor -1 (CAF1) (Stillman, 1986).

Figure 1.3

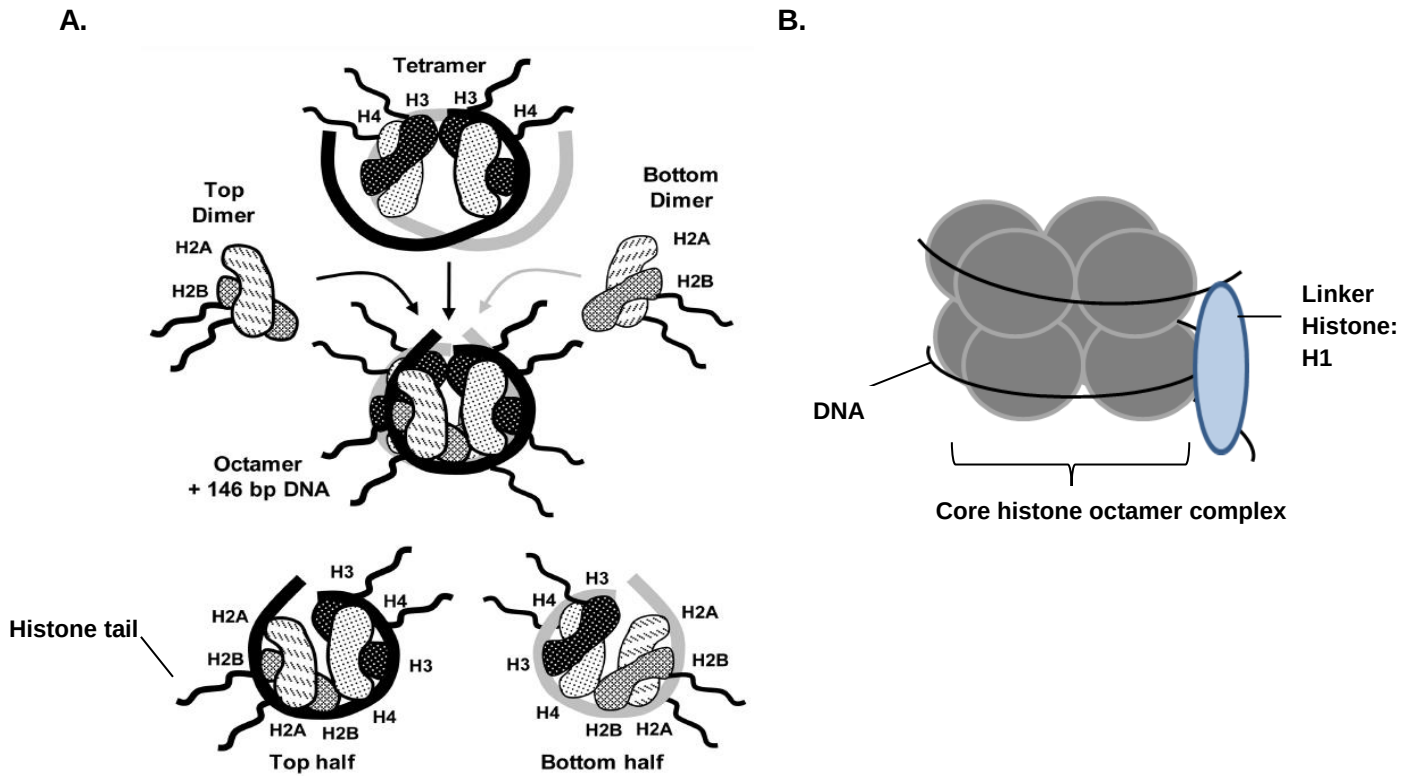


Figure 1.3 - Nucleosome structure. [A] **Histone octamer.** Nucleosome is formed by the wrapping of 145-260 bp DNA around a H3/H4 tetramer and a H2A/H2B dimer. N-terminal tails of histones protrude from the nucleosome forming a regulatory module. [B] **Core and linker histones.** Linker histone H1 is outside the core octamer and interacts with the linker DNA. [A is adapted from (review-Bonifer and Cockerill, 2011)]

1.2.4 Histone variants

Each histone protein has various isoforms and these are thought to serve specific functions at different chromatin compartments providing a layer of variation that causes differences in the overall structure of chromatin (review-Ausio, 2006). For example, transcriptionally active genes are associated with the histone H3 variant H3.3, whose exchange is possible with the help of the histone chaperone histone regulator A (HIRA) independently from replication (Ahmad and Henikoff, 2002; Tagami *et al.*, 2004). Another H3 variant, centromere protein (CENP) was linked to the assembly of the kinetochore and is exclusively found at centromeric regions (Palmer *et al.*, 1991; Przewloka *et al.*, 2011; Saitoh *et al.*, 1992). H2A variants also play distinctive roles in chromatin. For instance, H2AZ was mainly found at the promoters of genes (Guillemette *et al.*, 2005). H2AX was found to be essential in DNA double-strand breaks, replication, apoptosis, meiosis and recombination (Chen *et al.*, 2000; Mahadevaiah *et al.*, 2001; Rogakou *et al.*, 2000; Ward and Chen, 2001). MacroH2A was shown to be concentrated in the inactive X chromosome of females (Costanzi and Pehrson, 1998). Moreover, testis specific variants of H2B were identified (Shires *et al.*, 1976; Zalensky *et al.*, 2002). As opposed to core histones, variants of linker histones are not well characterised. Linker histone variant H1T was associated with spermatogenesis and some other isoforms were found in different species (Seyedin and Kistler, 1980).

1.2.5 Chromatin remodeling

Machineries involved in vital nuclear activities such as replication, damage repair or transcription need to have physical access to DNA in order to perform their functions. This can be achieved in a highly coordinated manner by numerous chromatin modifiers [explained in further sections] and chromatin remodelers which re-position, re-configure or eject nucleosomes (review-Cairns, 2009). Chromatin remodelers are multi-subunit complexes containing factors with an ATPase activity that disrupt interactions between nucleosomes, DNA and other chromatin proteins (review-Hargreaves and Crabtree, 2011). Four major classes of remodelers were defined based on the yeast ATPase homology; SWI/SNF, INO80/SWR1, ISWI and CHD families. Members of the SWI/SNF family mostly function at active gene promoters (Euskirchen *et al.*, 2011; Sudarsanam *et al.*, 2000) whereas INO80 remodelers assist homologous recombination, replication and damage repair (Shen *et al.*, 2000; West, 1997; Wu *et al.*, 2007). On the other hand, ISWI complexes are associated with inactive gene promoters where they re-organise nucleosomes in an unfavourable manner so that sequence specific activators cannot bind (Corona *et al.*, 2002; Whitehouse and Tsukiyama, 2006). Finally, CHD family members are known to aid the deposition of H3.3 in active genes (Gaspar-Maia *et al.*, 2009; Konev *et al.*, 2007).

1.3 Histone Modifications

1.3.1 General information

The key layer regulating chromatin structure is post-translational modifications of histones by nuclear enzymes, which mainly act on lysine (K), arginine (R) or serine (S) residues of histones (review-Bannister and Kouzarides, 2011; review-Kouzarides, 2007a). Transgenic models, mass spectrometry analyses and the development of chromatin immunoprecipitation (ChIP) techniques (Ebralidse *et al.*, 1993; Orlando *et al.*, 1997) led to their identification and allowed scientists to study their function. These biochemical alterations include acetylation, methylation, phosphorylation, ubiquitylation, sumoylation, ADP ribosylation, deiminitaion and proline isomerization. They typically occur at the flexible N-terminal tails of core histones but some may also take place at their globular domains. Knowledge about linker histone modifications is as yet rather limited. Phosphorylation is the primarily identified modification for linker histone H1 and it was correlated with a relaxed chromatin structure (Herrera *et al.*, 1996). Some other H1 modifications including acetylation and methylation have been described recently but their functions are not well characterised (Garcia *et al.*, 2004; Wisniewski *et al.*, 2007). Throughout this thesis, most attention will be paid to acetylation and methylation of the N-terminal tails of the core histones.

Each core histone modification is thought to play a role in chromatin mediated gene regulation either via affecting the electrostatic or hydrophobic status of histones and causing their structural perturbation or providing binding sites for chromatin associated proteins. In general, most important histone modifications were proven to be reversible and this allows dynamism in the regulation of chromatin under different conditions. The fact that there are many different synergistic or antagonistic modifications possible led to the hypothesis of the “histone code” which addresses the presence of different modification patterns for different chromatin states (review-Jenuwein and Allis, 2001). Thus, histone modifications as well as DNA methylation are thought to provide the molecular basis for epigenetic inheritance of chromatin states. Such epigenetic inheritance can be observed in somatic cells upon cellular division of a particular cell lineage or in the germ line from one generation to the next, a phenomenon known as “genomic imprinting” (review-Li and Sasaki, 2011; review-Probst *et al.*, 2009).

A graphical representation of the most common histone modifications along with their responsible enzymes is given in figure 1.4. Also, the effect of common histone modifications on transcription is given in table 1.1.

Figure 1.4

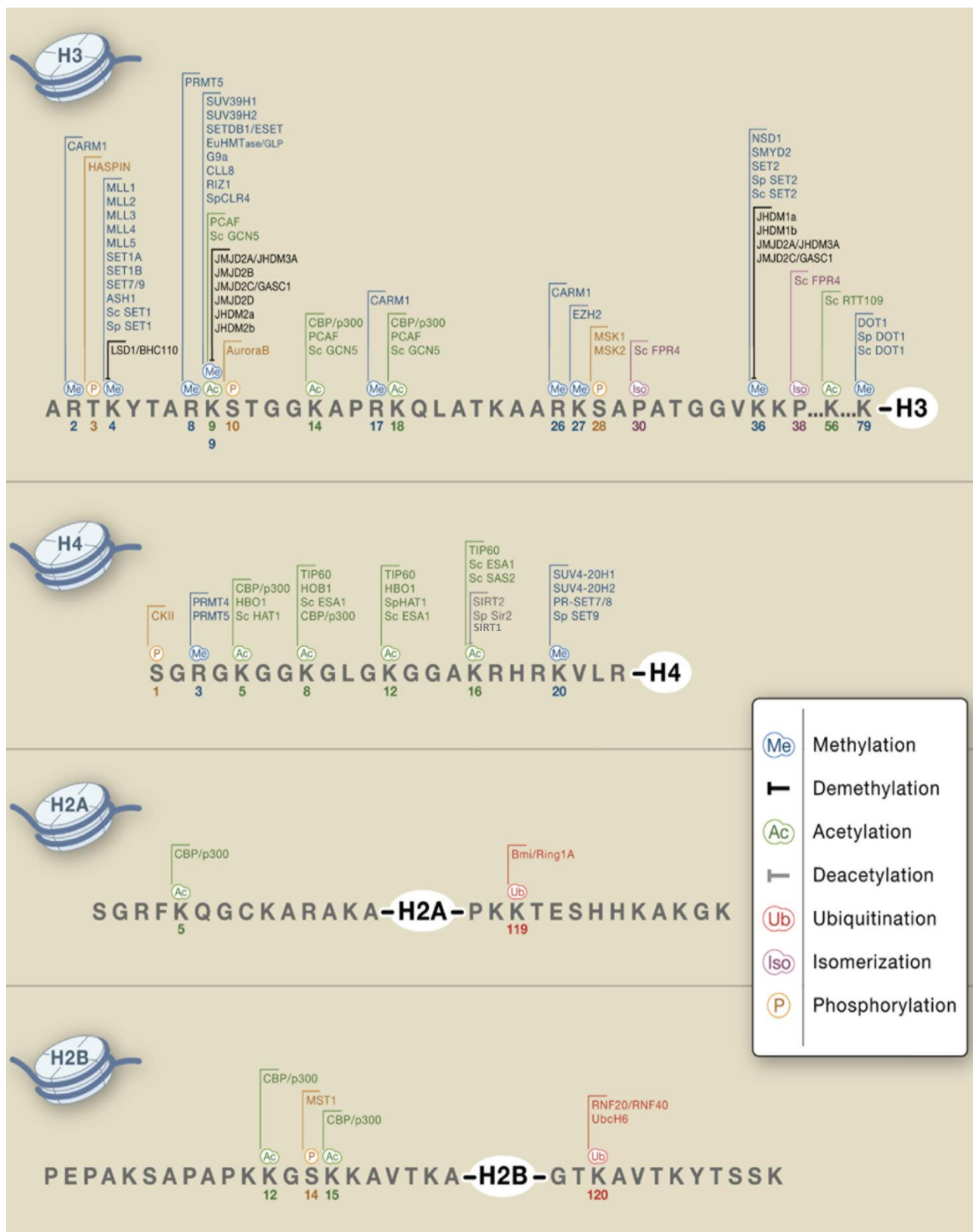


Figure 1.4 – Most common histone modifications and their primary responsible enzymes.

Yeast homologues start with a prefix “Sc” or “Sp” [Adapted from (snapshot-Kouzarides, 2007b)]

Table 1.1

Histone modification	Role in transcription	Modified residues
<i>Acetylation</i>	activation	H3(K9, K14, K18, K56)
		H4 (K5, K8, K12, K16)
		H2A
		H2B (K5, K15, K16)
<i>Phosphorylation</i>	activation	H3 (S10)
<i>Methylation</i>	activation	H3 (K4, K36, K79)
	repression	H3 (K9, K27)
		H4 (K20)
<i>Ubiquitination</i>	activation	H2B (K120 or K123*)
	repression	H2A (K119)
<i>Sumoylation</i>	repression	H3 (?)
		H4 (K5, K8, K12, K16)
		H2A (K125)
		H2B (K5, K15, K16)

Table 1.1 – Common histone modifications and their effect on transcription.

*K120 is later referred as K123 in the literature. [Adapted from (textbook-Allis *et al.*, 2007)]

1.3.2 Acetylation of lysines

1.3.2.1 Mechanism of action

All histones can be acetylated at various N-terminal lysines (K) and regardless of the residue where this modification takes place, acetylation usually correlates with the transcriptionally active states of chromatin (Allfrey *et al.*, 1964; Hebbes *et al.*, 1988; Shahbazian and Grunstein, 2007). On the other hand, transcriptionally repressed heterochromatin was generally shown to be hypoacetylated (Halleck and Gurley, 1981). The fundamental fact thought to underlie this phenomenon is that the negatively charged acetyl groups (CH_3CO^-) are reducing the affinity of positively charged histones towards DNA by neutralising lysine's negative charge, and therefore loosening the chromatin structure providing more access to the transcription machinery (Hong *et al.*, 1993; Sealy and Chalkley, 1978). In addition, acetylated histones primarily attract bromodomains, a common sub-domain of chromatin-remodeling complexes (review-Mujtaba *et al.*, 2007). Strikingly, the SWI/SNF remodeling complex was shown to be directly recruited by acetylated histones (Hassan *et al.*, 2002). PHD fingers can also recognise acetylated histones. One example is the DPF3b protein which is a component of the BAF chromatin-remodeling complex, a member of the SWI/SNF family in mammals (Zeng *et al.*, 2010). Histone acetylation also takes place in the cytoplasm and this has been linked to the nuclear import of histones and assembly into chromatin via CAF1 (Jackson *et al.*, 1976; Verreault *et al.*, 1996).

Acetylation could occur at a number of histone residues and acetylation marks typically work synergistically for the activation of transcription. This implies a cumulative effect of acetylated residues for loosening the chromatin structure. Among these many acetylated residues; however, H4K16 was shown to have a strong effect by itself for the disruption of tight chromatin and thereby activating gene expression (Dion *et al.*, 2005; Shogren-Knaak *et al.*, 2006). Another important residue is certainly H3K9 because its acetylation prevents a subsequent methylation, which acts as a strong repressor of transcription [see section 1.3.3.4] (Czermin *et al.*, 2001; Mottus *et al.*, 2000).

Histone acetylation is catalysed by histone acetyltransferases (HATs). On the other hand, counteracting histone deacetylases (HDACs), which are typically linked to transcriptional inactivation, can remove acetyl groups from histones providing a tight control mechanism on this prominent modification. In many reported cases, the expression and activity of HATs and HDACs are regulated in response to many different cellular conditions including cell cycle regulation, differentiation, apoptosis and DNA repair (review-Sengupta and Seto, 2004). Importantly, both HATs and HDACs can target non-histone substrates as well and these include transcription factors. Some of these enzymes may also target cytoplasmic

proteins, but only their actions in relation to chromatin and transcription will be covered within the context of this thesis. Because these enzymes primarily function in protein complexes, it is generally difficult to determine which one is responsible for a specific effect. Still, it is possible to at least determine which enzyme is required for a given nuclear process (review-Bannister and Kouzarides, 2011). Therefore, histone acetylation will be explained in a categorised manner based on the structural homologies of evolutionarily well-conserved HATs and HDACs and their functional implications.

1.3.2.2 Histone acetyltransferases

Histone acetyltransferases (HATs) can transfer an acetyl group from acetyl co-enzyme A to lysine (K) residues of all types of histones [Figure 1.5]. HATs, which usually interact with transcriptional activator complexes, can be classically categorised into four major groups based on their homology (review-Shahbazian and Grunstein, 2007; review-Sterner and Berger, 2000):

GNAT (Gcn5-related N-acetyltransferase) family

GCN5 (general control non-derepressible 5) was identified in *Tetrahymena thermophila* as the first transcription linked HAT and has conserved isoforms in mammals (Brownell *et al.*, 1996). Studies from yeast revealed that GCN5 has a preferential activity towards Histone H3 and can acetylate nucleosomal histones only in the presence of transcriptional activator complexes such as SAGA and ADA (Grant *et al.*, 1997; Kuo *et al.*, 1996). Among these, SAGA seems to have a preference for H3K9 residues (Grant *et al.*, 1999). Another enzyme showing homology with GCN5 is PCAF, which was shown to interact with transcriptional co-activators P300 and CREB-binding protein (CBP) in human cells (Yang *et al.*, 1996). Like GCN5, PCAF also primarily acts on H3 lysines (Schiltz *et al.*, 1999). Being first identified in *Saccharomyces cerevisiae*, HAT1 and HAT2 are other enzymes within this family and they act directly on free (non-nucleosomal) H4 histones and are therefore implicated in chromatin assembly at replication forks and telomeres (Ruiz-Garcia *et al.*, 1998). There are several other HATs from the GNAT family (i.e. ELP3 and HAP2) showing similar activities.

MYST (MOZ, YBF2, SAS2p, TIP) family

Unlike the GNAT family, these HATs mostly act on histone H4 lysine residues (review-Sapountzi and Cote, 2011). Monocytic leukemia zinc finger (MOZ) protein has been linked with nuclear receptor co-activator TIF2 (transcription intermediary factor 2) as well as the Runt domain transcription factor RUNX2 in mammals (Carapeti *et al.*, 1998; Pelletier *et al.*, 2002). Another MYST acetyltransferase, HBO1 (histone acetyltransferase binding to ORC1), was shown to interact with the origin recognition complex ORC1 in human cells providing a

link between histone acetylation and DNA replication (Iizuka and Stillman, 1999). Having a preference towards H4K16 residues in mammals (Taipale *et al.*, 2005), MOF (*Drosophila* males absent on the first, homologue) is one of the most important HATs. MOF regulates P53 dependent transcription by directly acetylating the DNA binding domain of P53 (Li *et al.*, 2009). MOF was also shown to interact with the DNA-dependent protein kinase catalytic subunit (DNA-PKcs), a protein involved in DNA repair (Sharma *et al.*, 2010). Another important phenomenon involving MOF is dosage compensation in *Drosophila* where its activity is needed for enhancing the transcription levels of the male X chromosome (Kelley *et al.*, 1995). Being the most studied MYST enzyme, TIP60 (Tat-interactive protein 60) is known to aid transcription in mammals by forming a complex with TRAPP (transcriptional adaptor protein) (Ikura *et al.*, 2000). TIP60 also forms a stable complex with the DNA damage sensor protein ATM (*Ataxia telangiectasia* mutated) whose direct acetylation is needed for its kinase activity targeting H2AX – a hallmark of the DNA damage response (Sun *et al.*, 2005).

P300/CBP family

These previously well-established, closely related transcriptional co-activators also carry a HAT activity domain (Bannister and Kouzarides, 1996; Ogryzko *et al.*, 1996). *In vitro* assays showed that human P300/CBP family HATs can transfer acetyl groups to all histones almost equally well (Schiltz *et al.*, 1999). Their specific action on H3K56 was recently shown to be linked with DNA repair but the exact mechanism is yet to be characterised (Das *et al.*, 2009).

Nuclear receptor co-activators

Hormonal signals may also play an active role in the chromatin acetylation by activating a specific subgroup of HATs known as receptor co-activators. A major example is the steroid receptor co-activator -1 (SRC1 or P160 or NCo-A1). SRC1 itself has HAT activity (preferentially on H3K9 residues) that is ligand dependent but it may also form complexes with P300/CBP and PCAF (P300/CBP associated factor) (Kamei *et al.*, 1996; Schubeler *et al.*, 2000; Spencer *et al.*, 1997; Vaquero *et al.*, 2004). Other well-known members of this family are nuclear receptor co-activator ACTR and transcriptional intermediary factor TIF2 (review-Sterner and Berger, 2000).

Figure 1.5

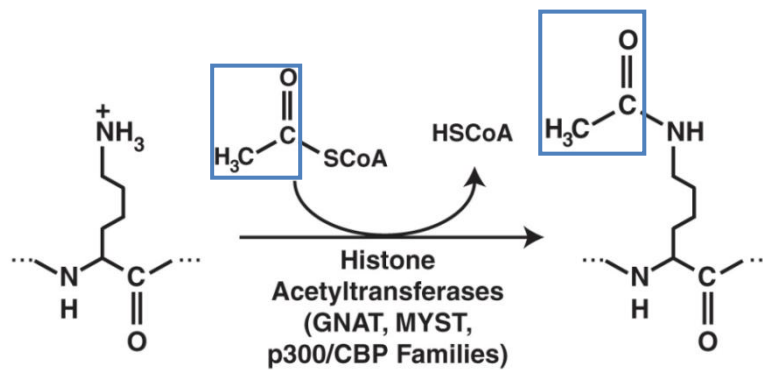


Figure 1.5 – Histone acetylation mechanism. HATs transfer an acetyl group (in blue box) from acetyl co-enzyme A (SCoA) to the ϵ -amino group of lysine (K) residues and this neutralises the positive charge. [Adapted from (review-Cyr and Domann, 2011)]

1.3.2.3 Histone deacetylases

Histone deacetylases (HDACs) are thought to repress transcription by removing the negatively charged acetyl group and restoring histone affinity for DNA back to native levels. Removal of the acetyl group allows other subsequent histone modifications at key histone residues, mainly methylation – which can lead to transcriptional repression (review-Martin and Zhang, 2005). HDACs were also shown to be interacting with numerous transcriptional repressor complexes. Broadly speaking, the preferences of HDACs towards specific histones or lysine residues are not really well characterised and they also target non-histone proteins (review-Yang and Seto, 2007). HDACs fall into four major groups based on their phylogeny and functional implications (Bjerling *et al.*, 2002; Gregoret *et al.*, 2004). HDAC class I, II and IV use a redox-active metal [Zn^{2+} or Fe^{2+}] in order to facilitate the removal of acetyl groups. In contrast to the other three classes, class III HDACs (sirtuins) need a NAD⁺ molecule for their deacetylation activity (Landry *et al.*, 2000). Figure 1.6 presents a simplified version of these biochemical reactions.

More detailed information about HDACs and their functions are given as the following:

HDAC class I

This group includes HDACs -1, -2, -3 and -8, which are nuclear and show homology with yeast (*Saccharomyces cerevisiae*) transcriptional regulator deacetylase RPD3 (review-de Ruijter *et al.*, 2003). Their activity is usually dependent on their interaction with other proteins. For example, HDAC1 dimerises with HDAC2 to form the catalytic core of the well-established transcriptional co-repressor complexes: SIN3 (switch independent 3), NuRD (nuclear remodelling and deacetylase) and CoREST (co-repressor of REST) (Xue *et al.*, 1998; You *et al.*, 2001; Zhang *et al.*, 1997). HDAC3, on the other hand, serves as the catalytic subunit of the N-CoR (nuclear receptor co-repressor) and SMRT (silencing mediator of retinoic acid and thyroid hormone receptor) co-repressor complexes (Heinzel *et al.*, 1997; Kao *et al.*, 2000). These function collaboratively with hormones in response to physiological stimuli. To name a few; unliganded retinoic acid receptor (RAR), thyroid hormone receptor (TR), antagonist-bound estrogen and progesterone receptors all interact with the SMRT complex, which in turn recruits the SIN3-HDAC complex in order to achieve silencing of relevant genes (review-Ng and Bird, 2000). Also, it is remarkable that the DNA methyltransferase enzyme DNMT3a was found to be co-functioning with HDACs -1 and -2 (Fuks *et al.*, 2001). Recent findings suggest specific action of HDACs -1 and -2 on H4K16 and H3K56 as a consequence of the DNA damage response (Miller *et al.*, 2010). HDAC3 was shown to be more specific to H3K9, K14; H4K5; and H4K12 residues via knockout studies, as its loss increased the global acetylation levels of these residues (Bhaskara *et al.*,

2010). There is not much knowledge in the literature so far about the direct chromatin-related effects of HDAC8 but it was indirectly linked to the inactivation of CREB (c-AMP response element binding) mediated gene transcription by stabilising the PP1 phosphatase, which targets CREB and causes its inactivation (Gao *et al.*, 2009).

HDAC class II

Enzymes within this group have homology with the yeast deacetylase/transcriptional repressor HDA1 (histone deacetylase 1). They are mostly cytoplasmic but can also be found in the nucleus. This group can be further sub-divided; class IIa includes HDACs -4, -5, -7, -9 and class IIb consists of HDACs -6, -10 (review-de Ruijter *et al.*, 2003; review-Yang and Seto, 2003). Chromatin related actions of HDACs -4, -5 and -7 is dependent on their interaction with the HDAC3/SMRT/N-CoR complex (Fischle *et al.*, 2002). All class IIa HDACs including HDAC9 were shown to regulate MEF2, an essential transcription factor for muscle differentiation (McKinsey *et al.*, 2000; Zhou *et al.*, 2001). In terms of class IIb, there is not much known about the nuclear functions of HDAC6 apart from its interaction with HDAC11 (Gao *et al.*, 2002). HDAC10, on the other hand, was shown to deacetylate transcription factors PAX3 (paired box 3) and KAP2 (keratin associated protein 2) contributing to melanogenesis (Lai *et al.*, 2010).

HDAC class III (Sirtuins)

Sirtuins were the last discovered but perhaps most studied HDACs participating in numerous cytoplasmic and nuclear pathways with broad impacts on aging, cancer, diabetes and neurodegenerative diseases (review-Haigis and Sinclair, 2010). There are seven members of the sirtuin family; SIRT 1-7. These are all orthologues of the yeast transcriptional silencer/deacetylase SIR2 (review-Blander and Guarente, 2004; review-Vaquero, 2009). Having the highest homology with yeast SIR2, SIRT1 is the most studied sirtuin and has the broadest target spectrum. Its activity is essential within the chromatin context. SIRT1 knockdown elevated global acetylation levels of H4K16 and H3K9 implying its specificity towards these key residues. Notably, its knockdown also reduced heterochromatin marks H3K9-tri-methylation (H3K9me3) and H4K20-mono-methylation (H4K20me1) suggesting an important role of SIRT1 in heterochromatin formation (Vaquero *et al.*, 2004). The same study also reports direct activity of SIRT1 on the K26 residue of linker histone H1, which is rapidly deposited into chromatin with SIRT1 recruitment. Furthermore, it was reported that SIRT1 can directly recruit and activate the H3K9 specific histone methyltransferase SUV39H1, whose activity can be essential for the establishment of heterochromatin mediated gene repression in particular contexts [see section 1.3.3.4] (Vaquero *et al.*, 2007). SIRT1 also deacetylates and regulates the activity of many crucial transcription factors including FOXO family factors (Motta *et al.*, 2004), the major cell cycle regulator P53 (Vaziri *et al.*, 2001), and

the DNA repair factor KU70 (Cohen *et al.*, 2004). SIRT2 is mainly cytoplasmic but also functions in the nucleus particularly immediately before mitosis, primarily acting on H4K16 and to a lesser extent on H3K9 residues (Vaquero *et al.*, 2006). Although its primary location is in mitochondria, SIRT3 can be targeted to the nucleus and primarily deacetylate H4K16 and H3K9 residues probably on a small subset of genes (Scher *et al.*, 2007). Mitochondrial SIRT -4 and -5, on the other hand, have not yet been linked to any chromatin related functions so far. All mitochondrial sirtuins (SIRT3-5) are known to play essential roles in the biogenesis of mitochondrial NAD⁺ and generation of reactive-oxygen species (ROS) as well as signalling during apoptosis (review-Haigis and Sinclair, 2010). SIRT6 has a role in promoting DNA end resection, a crucial step in double-strand break repair by deacetylating DSB resection protein CTIP (Kaidi *et al.*, 2010). SIRT6 was also observed in telomeric regions, where it primarily targets H3K9 residues (Michishita *et al.*, 2008). SIRT7 was reported to deacetylate and activate RNA polymerase-I directly (Ford *et al.*, 2006). Notably, all sirtuins are likely to be post-translationally modified (i.e. phosphorylation) or regulated by protein-protein interactions (e.g. AROS, DBC1 and Necdin, as described for SIRT1) (Hasegawa and Yoshikawa, 2008; Kim *et al.*, 2007; Kim *et al.*, 2008; Sasaki *et al.*, 2008).

HDAC class IV

This group contains only one enzyme; HDAC11, which shares structural and functional homology both with yeast RPD3 and HDA1. This enzyme was not found in any of the classical HDAC transcriptional repressor complexes (SIN3, SMRT, N-CoR) (Gao *et al.*, 2002). Recently, it was found to modulate the stability of DNA replication licensing factor CDT1 (Glozak and Seto, 2009).

Figure 1.6

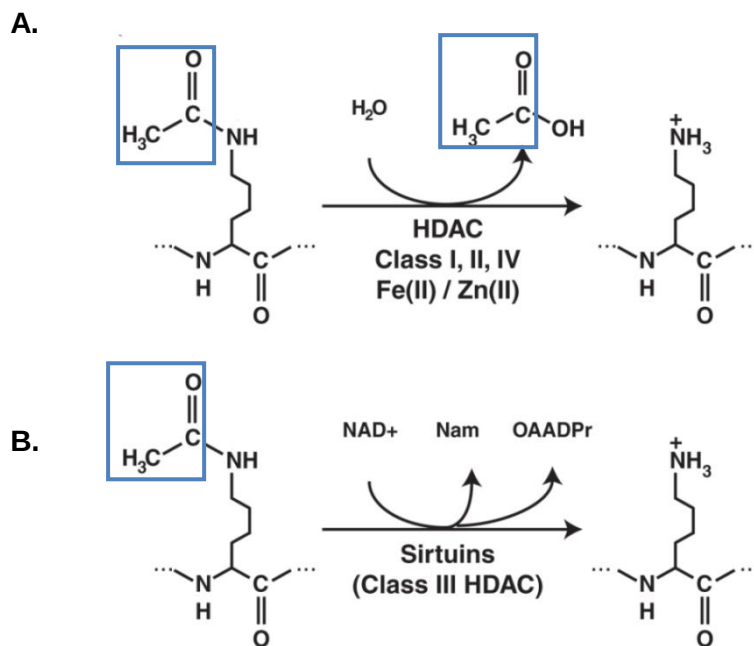


Figure 1.6 – Histone deacetylation mechanism. [A] Biochemical mechanism for HDAC class I, II and IV. These classical HDACs utilise a redox-active metal [Zn^{2+} or Fe^{2+}] in order to catalyse the hydrolysis of acetyl group from lysine residues. [B] HDAC class III (sirtuins) deacetylation mechanism. Sirtuins transfer the acetyl-group from the substrate to the NAD^+ molecule and generate O-acetyl-ADP-ribose (OAADPr) and nicotinamide (Nam). [Acetyl groups are in blue box. This figure is adapted from (review-Cyr and Domann, 2011).]

1.3.2.4 Histone deacetylase inhibitors

The discovery of diverse roles for the HDACs which impact on the regulation of chromatin maintenance as well as many other key subcellular events including cell differentiation and proliferation led to the development of HDAC specific chemical inhibitors (review-Hahnen *et al.*, 2008; review-Rosato and Grant, 2005; review-Xu *et al.*, 2007b). Classical HDAC inhibitors competitively target HDAC classes I, II and IV by a zinc binding moiety that interacts with the catalytic site. Perhaps the most well-known examples for this group are hydroxamic acid TSA (trichostatin A) and SAHA (suberoylanilide hydroxamic acid) (Richon *et al.*, 1996; Yoshida *et al.*, 1990). Both of these drugs were used in numerous experimental approaches in understanding the mechanisms of chromatin regulation and also showed significant anti-cancer and neuro-protective effects with therapeutic potential (review-Hahnen *et al.*, 2008; review-Marks and Xu, 2009). Class III HDACs (sirtuins) are generally not affected by the zinc binding inhibitors and can typically be inhibited in a non-competitive manner by NAD⁺ analogues or sirtuin structure specific agents (review-Cen, 2010). The most prominent sirtuin inhibitors are nicotinamide, suramin, cambinol, indole, splitomicin and salermide (review-Alcain and Villalba, 2009). In this thesis, a gene expression enhancing role for the sirtuin inhibitor nicotinamide will be presented.

Nicotinamide (Nam) is the amide form of Vitamin B3 and an endogenous sirtuin inhibitor [Figure 1.7A] (Avalos *et al.*, 2005; Bitterman *et al.*, 2002). It is actually a by-product in the metabolic cycle of nicotinamide adenine dinucleotide (NAD⁺), which is an essential coenzyme involved in the cellular redox reactions (review-Belenky *et al.*, 2007). NAD⁺ can be synthesised *de novo* from tryptophan or recycled in four steps from nicotinamide via the salvage pathway [Figure 1.7B]. Here, sirtuins and a number of NAD⁺ metabolising factors including ADP ribose transferase (ART), poly-ADP-ribose polymerase (PARP) and surface glycoprotein CD38 start the cycle with the production of nicotinamide, which is then converted to nicotinamide mononucleotide by NAMPT (also known as PBEF) and recycled back to NAD⁺ via the activity of isozymes NMNAT1, NMNAT2 and NMNAT3; in the nucleus, golgi apparatus and mitochondria, respectively (Berger *et al.*, 2005; Revollo *et al.*, 2004; Rongvaux *et al.*, 2002). Consistently, exogenous nicotinamide increases NAD⁺ levels and plays a significant role in the mitochondrial respiratory electron transport chain (Lin and Guarente, 2003; Magni *et al.*, 2004; Sadanaga-Akiyoshi *et al.*, 2003).

Sirtuins transfer acetyl groups to the ADP-ribose of NAD⁺ and hence hydrolyse NAD⁺ releasing O-acetyl-ADP ribose and nicotinamide (Tanner *et al.*, 2000). When present in excess levels, free nicotinamide can reversibly bind to the releasing pocket in the sirtuin enzyme and prevent necessary conformational changes for the deacetylation reaction

[Figure 1.7C]. Primary mammalian sirtuin targets of nicotinamide are considered to be SIRT1 and SIRT2 (Bitterman *et al.*, 2002; Marcotte *et al.*, 2004; Suzuki and Koike, 2007; Suzuki *et al.*, 2006). However, some studies also suggested nicotinamide's inhibitory actions on SIRT3 (Yang *et al.*, 2010) and SIRT6 (Kaidi *et al.*, 2010).

Various observations in different model organisms and human cells reported an increase in histone acetylation (particularly at H4K16, H3K56 and H3K9 residues) and decrease in histone methylation (i.e. H3K9me) after RNAi based sirtuin inhibition or nicotinamide treatment (Choy *et al.*, 2011; Das *et al.*, 2009; Guillemette *et al.*, 2011; Tjeertes *et al.*, 2009; Vaquero *et al.*, 2007; Vaquero *et al.*, 2004). Importantly, a gene activating effect based on the chromatin influencing potential of nicotinamide has been revealed on the heterochromatinised *FMR1* gene within the context of Fragile X syndrome [see section 1.8.3.3] (Biacsi *et al.*, 2008). Interestingly, it was also reported to impair apoptosis in rat neurons by a mechanism that involves the activation of the protein-kinase B AKT1 pathway followed by the inhibition of the apoptotic transcription factor FOXO3a; despite the fact that FOXO3a is actually destabilised by SIRT1 (Chong *et al.*, 2005; Chong *et al.*, 2004; Wang *et al.*, 2011a). Although the mechanism behind this phenomenon is not clearly elucidated yet, many studies emphasised nicotinamide's anti-apoptotic effects characterised by increased stability of mitochondrial membrane potential and decreased cytochrome release, as well as decreased caspase and PARP1 (poly (ADP-ribose) polymerase 1, a hallmark of the DNA damage response) activity (Chong *et al.*, 2005; Chong *et al.*, 2004; Lin *et al.*, 2000; review-Maiese *et al.*, 2009). Consistently, nicotinamide is also considered to be an effective agent to alleviate symptoms of oxidative cellular stress by decreasing the externalisation of phosphatidylserine residues as well as ensuring genomic integrity via an as yet unclarified mechanism (Crowley *et al.*, 2000; Li *et al.*, 2006a; Lin *et al.*, 2000). Furthermore, nicotinamide acts as a pro-inflammatory cytokine blocker with potential effects on major histocompatibility complexes (Fukuzawa *et al.*, 1997; Ungerstedt *et al.*, 2003). Considering these anti-apoptotic and anti-inflammatory roles, nicotinamide has been implicated as a potential therapeutic agent in Type-I diabetes, Alzheimer's and Parkinson's diseases (Crino *et al.*, 2005; Green *et al.*, 2008; Jia *et al.*, 2008; Olmos *et al.*, 2006; Vague *et al.*, 1987).

Nicotinamide is generally considered as a food supplement and has recently been investigated in the prevention of Type-I diabetes in at risk individuals (review-Knip *et al.*, 2000). IC₅₀ (half of the maximum inhibitory dose) for its *in vitro* inhibitory effect on SIRT1 was calculated as ~0.25mM (Sanders *et al.*, 2009). However, many studies reported sufficient inhibition at a dose of 1-30 mM in cell culture (Bitterman *et al.*, 2002; Marcotte *et al.*, 2004; Tjeertes *et al.*, 2009; Vaquero *et al.*, 2007). On the other hand, LD₅₀ (half of single lethal

dose) value on mice is 2.5 g/kg when administered intravenously (Hoffer, 1967). Studies addressing potential effects in diabetes and neurodegenerative diseases revealed a dose range varying from 100 – 1000 mg/kg in mice in order for nicotinamide to exert its function significantly and safely (Ieraci and Herrera, 2006; review-Knip *et al.*, 2000; O'Brien *et al.*, 2000). The compound effectively crosses the blood-brain barrier and has a metabolic half-life of ~4.5 hours with a plateau level reached in 3 hours after the treatment (Hoane *et al.*, 2006; Spector and Kelley, 1979; Stratford and Dennis, 1994). Side effects include liver toxicity, weight changes and skin conditions with considerably low oncogenic potential (review-Knip *et al.*, 2000).

Conversely to nicotinamide function, numerous synthetic small molecules and bioavailable resveratrol were identified as sirtuin activators (Bemis *et al.*, 2009; Howitz *et al.*, 2003; Kaeberlein *et al.*, 2005; Milne *et al.*, 2007). However, recent experimental evidence suggests that earlier findings related to these activators including resveratrol appeared to be an experimental artefact (Beher *et al.*, 2009; Pacholec *et al.*, 2010). Rather, resveratrol seems to exert its function on the AMP activated protein kinase (AMPK) (Baur *et al.*, 2006; Beher *et al.*, 2009; Jager *et al.*, 2007; Zhang, 2006).

Figure 1.7

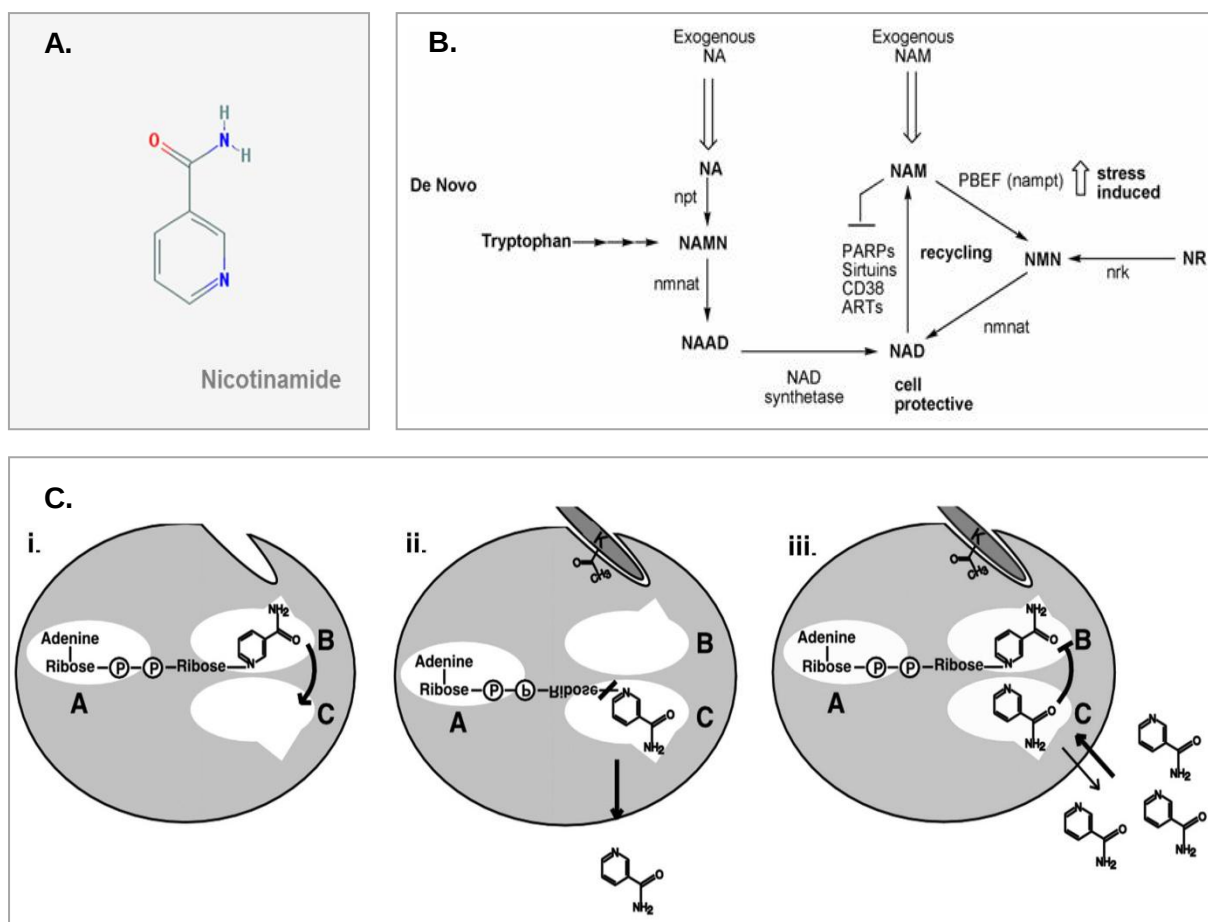


Figure 1.7 – NAD⁺ metabolism and sirtuin inhibition by nicotinamide. [A] Structure of nicotinamide. [B] Mammalian NAD⁺ metabolism via salvage pathway. NAD⁺ (nicotinamide adenine dinucleotide) can be synthesised *de novo* from tryptophan or can be recycled via the salvage pathway. Here, PARPs, sirtuins, CD38 and ARTs consume NAD⁺ and generate nicotinamide (NAM). This is then converted to NMN and subsequently to NAD⁺ via the activities of NAMPT and NMNATs, respectively. [NA indicates nicotinic acid; NAMN: nicotinic acid mononucleotide, NAAD: nicotinic acid adenine dinucleotide, NR: nicotinamide riboside, NMN: nicotinamide mononucleotide, NAM: nicotinamide, npt: nicotinic acid phosphoribosyltransferase, nmnat: nicotinic acid/nicotinamide mononucleotide adenyltransferase, nrk: nicotinamide riboside kinase, nampt: nicotinamide phosphoribosyltransferase, PBEF (NAMPT): pre-B-cell enhancing factor, PARPs: poly(ADP-ribose) polymerases, ART: ADP ribose transferase.] [C] Sirtuin inhibition by nicotinamide. (i) SIRT1 contains three pockets for the deacetylation reaction. Pocket A has the ADP ribose of NAD⁺, pocket B is occupied by nicotinamide in the absence of substrate. (ii) In the presence of substrate, nicotinamide is repositioned to pocket C and an acetyl group from acetyl-lysine to the ADP ribose of NAD⁺ is transferred by SIRT1, releasing O-acetyl ribose and nicotinamide. (iii) When free nicotinamide is in excess levels, it can occupy the pocket C preventing the repositioning and hence the deacetylation reaction. [Chemical structures PubChemCompound, <http://www.ncbi.nlm.nih.gov/pccompound>; B adapted from (review-Yang and Sauve, 2006) and C from (Bitterman *et al.*, 2002)]

1.3.3 Methylation of lysines

1.3.3.1 Mechanism of action

Unlike acetylation or any other modification, methylation appears more complex in how it might participate in chromatin mediated gene regulation. It is associated with both activation and repression of transcription – depending on the position of the methylated histone residue or the type of methylation. This covalent modification can take place on the side chains of both lysine (K) and arginine (R) residues, primarily on histones H3 and H4 (review-Kouzarides, 2007a; Murray, 1964).

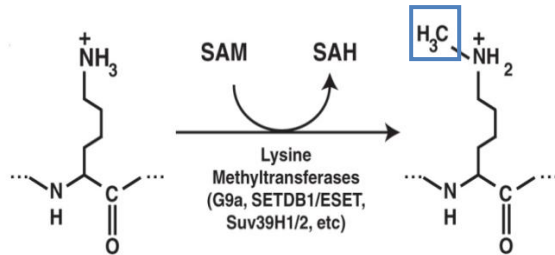
Lysine methylation is catalysed by histone lysine methyltransferases (HKMTs). Following the discovery of the first HKMT; SUV39H1 (Rea *et al.*, 2000), many more have now been identified. All of these enzymes are evolutionarily well conserved and share a SET domain as a catalytic site except the more recently discovered DOT1 (Min *et al.*, 2003). HKMTs transfer methyl groups ($R-CH_3$) to the ϵ -amino group of N-terminal lysines from cofactor S-adenosyl-L-methionine [Figure 1.8A]. Methylation does not alter the electrical charge but increases the hydrophobicity and may disrupt intra- and intermolecular hydrogen bonds. It is thought that this results in a conformational change of the histone tail, affecting its ability to hold DNA. Another outcome of this structural change is the creation of binding sites recognised by the ‘reader’ proteins; typically with chromo-, tudor- and PHD-repeat domains, which introduce activating or repressing effects on chromatin (review-Lee *et al.*, 2005b). Importantly, lysines can be mono-, di- or tri- methylated [Figure 1.8B]. This tri-modal pattern brings extra complexity in terms of the outcome of the methylation event.

Histone methylations are quite stable and they were thought to be irreversible for a long time; however, identification of histone lysine demethylases (HKDMs) revealed that this modification is dynamically regulated (review-Shi, 2007). The first HKDM was proven to be the nuclear amine oxidase homologue LSD1 (lysine specific demethylase 1) which catalyses lysine demethylation by a FAD⁺ (flavine adenine dinucleotide) dependent mechanism [Figure 1.8C] (Shi *et al.*, 2004). Next, few other demethylases with a distinct catalytic structure containing the Jumonji-C (JmjC) domain were identified. These enzymes remove methyl groups from lysine residues in a 2-oxoglutarate (2-OG) and Fe(II) dependent manner [Figure 1.8D] (Tsukada *et al.*, 2006).

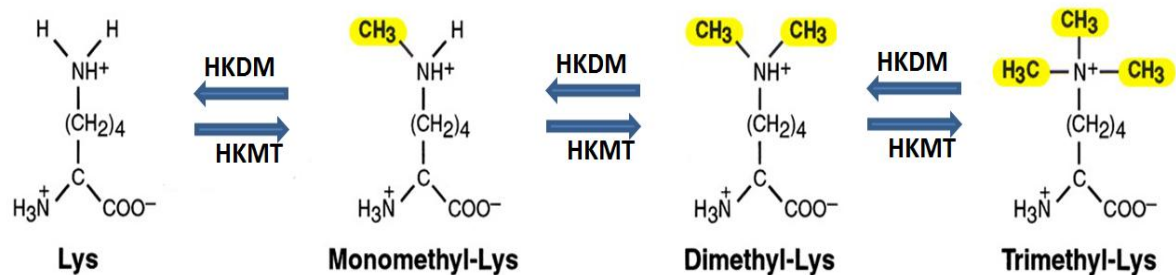
Unlike HATs and HDACs, enzymes responsible for the regulation of lysine methylation are generally highly specific to distinct histone residues. There are at least 24 histone methylation sites identified but three of them (i.e. H3K4, H3K27 and H3K9) will be introduced in more detail due to the context of this thesis.

Figure 1.8

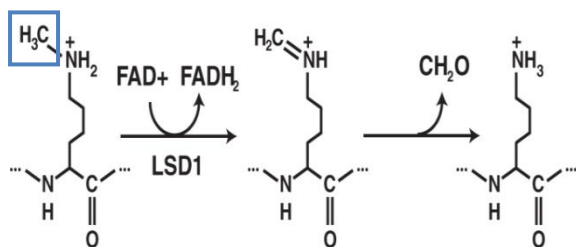
A.



B.



C.



D.

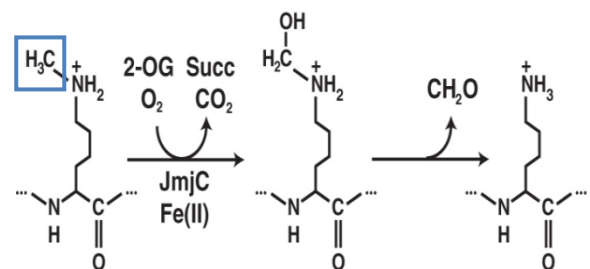


Figure 1.8 – Lysine methylation and demethylation. [A] Methylation of a lysine. HKMTs utilize S-adenosyl-L-methionine (SAM) as a methyl donor. [B] Lysines can be mono-, di- or tri- methylated. Methyl groups (marked with yellow), are transferred by histone lysine methyltransferases (HKMT) and can be removed via the activity of histone lysine demethylases (HKDM) [C] LSD1 demethylation. LSD1 hydrolyses methylated lysines in a FAD^+ (flavine adenine dinucleotide) dependent mechanism. [D] Demethylation by JmjC-domain HKDMs. JmjC (Jumonji) demethylases need 2-oxoglutarate (2-OG) and Fe^{2+} for their catalytic action. [Methyl groups are in blue box. A,C,D adapted from (review-Cyr and Domann, 2011), B adapted from (review-Zhang and Reinberg, 2001)]

1.3.3.2 H3K4 methylation

This methylation mark is generally correlated with transcriptionally active genes and is thought to demarcate euchromatic genes (textbook-Allis *et al.*, 2007; Litt *et al.*, 2001; Noma *et al.*, 2001). Studies addressing the mechanistic impact of this correlation were primarily carried out in *S. cerevisiae* (budding yeast), where the first H3K4 specific HKMT 'SET1' was identified (Briggs *et al.*, 2001). Subsequent observations in yeast revealed that di-methylated H3K4 occurs at the 5' regions of both inactive and active euchromatic genes, whereas tri-methylated H3K4 appears exclusively at active genes (Santos-Rosa *et al.*, 2002). The mechanistic link behind this phenomenon was proven to be the recruitment of SET1 through the initiating RNAPII, which is phosphorylated at the serine-5 residue of its carboxy-terminal domain (CTD) [see section 1.6.3.2 and figure 1.17 and 1.18] (Ng *et al.*, 2003). PAF1 (polymerase associated factor 1) complex, which regulates RNA metabolism, also interacts with SET1, probably implying a role in the early elongation complex (Krogan *et al.*, 2003). Moreover, H3K4 methylation can be recognised by chromatin remodelers such as CHD1 and NURF complexes which tighten the link between this methylation mark and transcriptional activation (Li *et al.*, 2006b; Sims *et al.*, 2005). Notably, a similar mechanism was also described for H3K36 methylation, which is enriched at 3' regions of transcriptionally active genes. H3K36 specific HKMT SET2 interacts with the elongating form of RNAPII, which is phosphorylated at serine-2 of its CTD domain (Xiao *et al.*, 2003). Importantly, H3K4 methylation marks were also detected at the 5' ends of human genes whereas methylated H3K36 residues were detected at 3' ends (Barski *et al.*, 2007; Liang *et al.*, 2004). This provides evidence for evolutionary conservation of this mechanism.

There are at least eight curated H3K4 specific HKMTs (review-Ruthenburg *et al.*, 2007): the 'mammalian mixed lineage leukemia' (MLL -1,-2, -3, -4) family of enzymes show homology with yeast SET1 and *Drosophila* Trithorax (TRX). MLLs primarily catalyse H3K4 methylation and their activity was shown to be crucial for HOX (homeotic box) gene activation (Milne *et al.*, 2002). Other H3K4 specific enzymes include structurally different ASH1, SMYD3, SET7/9 and Meisetz. ASH1 (*Drosophila* absent, small, homeotic discs, homologue) was shown to activate ultrabithorax transcription via di-methylating H3K4 residues and triggering chromatin remodelling in *Drosophila* (Beisel *et al.*, 2002). Another mammalian HKMT, SMYD3 (SET and MYND domain containing 3) trimethylates H3K4 residues and was reported to form a complex with RNAPII during the activation of homebox genes as well as some oncogenes (Hamamoto *et al.*, 2004). SET7/9 is a mono-methylating mammalian HKMT and was shown to activate transcription in genes involved in glucose-stimulated insulin secretion (Deering *et al.*, 2009; Wang *et al.*, 2001). Finally, the activity of Meisetz,

which is a tri-methylating HKMT, was shown to be essential for the progression of early meiotic prophase (Hayashi *et al.*, 2005).

H3K4 demethylation is catalysed dynamically either by LSD1 or JmjC demethylases JARID1A-D. LSD1, which associates with repressor complexes such as HDAC1/2 and CoREST, demethylates mono- and di- methylated H3K4 residues (Shi *et al.*, 2004; Shi *et al.*, 2005). On the other hand, JARID enzymes catalyse the demethylation of tri-methylated H3K4 residues (Seward *et al.*, 2007).

Another important feature of H3K4 methylation is its ability to 'cross-talk' with other modifications. For example, a study performed in human cell lines reported that H3K4 methylation can prevent H3K9 methylation by precluding the major H3K9 specific HKMT, SUV39H (Nishioka *et al.*, 2002; Wang *et al.*, 2001). Additionally, H3K4 methylation was shown to be positively regulated by H2BK123 ubiquitylation in budding yeast (Sun and Allis, 2002).

1.3.3.3 H3K27 methylation and polycomb system

Methylation of H3K27 residues is primarily linked to transcriptional silencing of euchromatic genes, playing an essential role in the establishment of facultative heterochromatin. Although recent genome-wide studies performed with human cells revealed that mono-methylation of H3K27 residues appear on transcriptionally active genes via an yet-unidentified mechanism, di- and tri- methylated H3K27s are still closely linked to transcriptionally inactive euchromatic genes (Barski *et al.*, 2007; review-Black and Whetstine, 2011; Mikkelsen *et al.*, 2007; Vakoc *et al.*, 2006). The silencing activity through H3K27 di- and tri- methylation involves the polycomb group (PcG) of proteins and their homologues. PcG proteins were previously identified as transcriptional repressors and were known to contribute to cellular memory and orchestrate the activity of HOX genes, which organize body segmentation and vertebrate development (review-Kennison, 1995; Lewis, 1978). Although this system has been overwhelmingly studied in *Drosophila*, main principles were also confirmed in mammals (van der Lugt *et al.*, 1994). The exact mechanism of PcG mediated gene silencing remained unknown until it was discovered that the PcG system exerts its effect by modifying chromatin via a specific HKMT activity on H3K27 residues. Two protein complexes known as PRC1 (Polycomb repressor complex 1) and PRC2 were found to be essential for this phenomenon (review-Hubblitz *et al.*, 2009; review-Otte and Kwaks, 2003). A diagram summarising the activity of PRC complexes is given in figure 1.9.

PRC2 complex comprises three proteins: enhancer of zeste (E[z], human homologue: EZH2], suppressor of zeste 12 (Su[z]12, human homologue: SUZ12) and extra sex combs (ESC, human homologue: EED). EZH2 has a SET domain protein and can di- or trimethylate H3K27 residues via its HKMT activity (Cao *et al.*, 2002; Czermin *et al.*, 2002; Muller *et al.*, 2002). SUZ12 has a stabilising effect on the protein complex and EED seems to guide EZH2 towards H3K27 residues (Kuzmichev *et al.*, 2004; Pasini *et al.*, 2004). Crucially, PRC2 was reported to interact with HDACs linking H3K27 methylation with hypoacetylation. One important example is the interaction of EED with the histone deacetylase SIRT1 (Kuzmichev *et al.*, 2005). Other repressive functions that the PRC2 complex accommodates include an indirect effect on DNA methylation via EZH2 and interaction with H3K4 specific demethylases (Pasini *et al.*, 2008; Vire *et al.*, 2006).

PRC1 complex has four components: polycomb proteins (Pc, human homologues CBX -2, -4, -6, -7, -8), posterior sex combs (PSC, human homologue: Bmi1), polyhomeotic (PH, human homologue: HPH) and RING finger protein 1 (RING1, human homologue: RING1). Polycomb proteins share chromodomains which recognise and bind to methylated H3K27 residues (Fischle *et al.*, 2003). Notably, some studies also suggest binding of polycomb proteins to methylated H3K9 residues (Bernstein *et al.*, 2006b). Polyhomeotic proteins seem to propagate polymerisation of PRC complexes via intramolecular interactions (Kim *et al.*, 2002). Such interactions result in a moderate level of chromatin compaction (Francis *et al.*, 2004; Grau *et al.*, 2011). Once recruited to the target region, PRC1 complex can ubiquitinate H2AK119 residues via the ubiquitin ligase RING1, whose activity seems to be stimulated by BMI1 (Cao *et al.*, 2005; Wang *et al.*, 2004). For long, the function of this core histone ubiquitination remained unclear until it was discovered that this modification holds RNAPII in a maturation state that is incompatible with efficient transcription at bivalent genes in mouse embryonic stem cells [see section 1.6.4 and figure 1.18] (Stock *et al.*, 2007). Still, the exact character of this unusual RNAPII configuration in relation to PRC1 needs to be elucidated. Interestingly, another study reported that H2AK119ub1 prevents the recruitment of FACT (chromatin transcription) complex, which is essential for nucleosome remodelling and hence proper elongation of RNAPII (Zhou *et al.*, 2008). These results fit well with the previous finding that the PRC1 complex inhibits the recruitment of SWI/SNF remodelling complexes (Shao *et al.*, 1999).

Polycomb response element(s) (PRE), which have various DNA sequence patterns, are the anchor sites for the recruitment of PRC complexes to these target genes (Simon *et al.*, 1993). PREs have been extensively studied in *Drosophila* within the HOX gene cluster, but their existence has been recently confirmed in human as well (Woo *et al.*, 2010).

Mechanistically, PREs are recognised by several motif-recognising DNA binding proteins (i.e., GAF, ZESTE, DSP1 [*Drosophila*] or YY1 [human]) which attract PRC complexes (review-Ringrose and Paro, 2007). Another important example where the PcG system is involved in is the X inactivation process, where the coating of the inactivated X chromosome with Xist RNA is accompanied by the methylation of H3K27 residues via the activity of the PRC2 complex (Plath *et al.*, 2003; Silva *et al.*, 2003). The PRC1 complex was also found in the inactivated X chromosome but its exact function remains to be investigated (Hernandez-Munoz *et al.*, 2005).

Demethylation of H3K27 residues is catalysed by two JmjC domain containing HKDMs; an X inactivation escapee (a gene that is not silenced by X chromosome inactivation) UTX (Ubiquitously transcribed X-chromosome) and JMJD3 (JmJC-domain-containing protein 3) (Hong *et al.*, 2007). Several reports stated that demethylation of H3K27 residues by UTX or JMJD3 relieves the expression of PcG repressed HOX genes and plays an important role in embryonic development (Agger *et al.*, 2007; Lan *et al.*, 2007). Targeting mechanisms of these HKDMs are not well characterised yet but they seem to have an important role in the process of transcriptional activation. Importantly, UTX co-localises with elongating RNAPII (Smith *et al.*, 2008). Moreover, both UTX and JMJD3 were reported to interact with the H3K4 specific MLL/SET1 HKMT complexes, suggesting a synergistic role with H3K27 demethylation and H3K4 methylation in the activation of transcription (De Santa *et al.*, 2007; Lee *et al.*, 2007b).

Figure 1.9

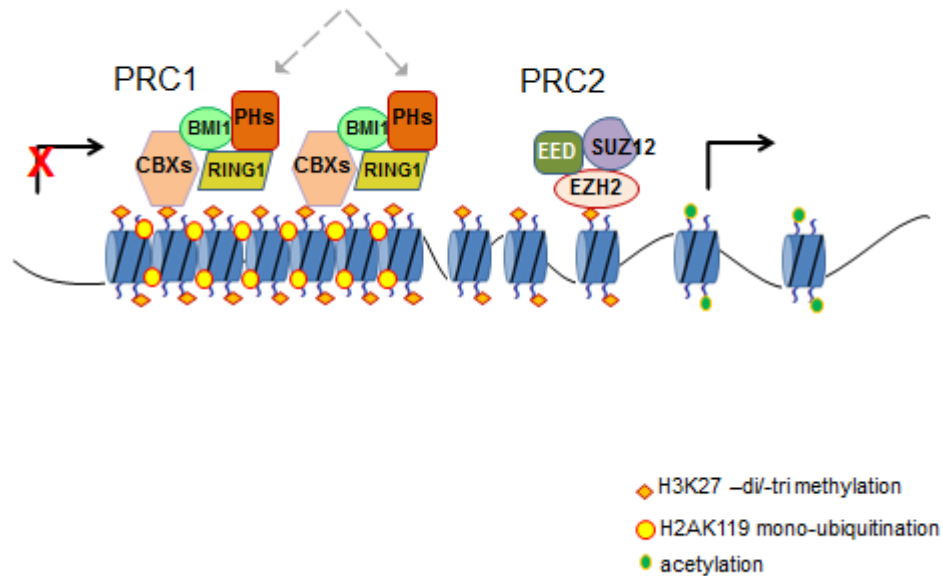


Figure 1.9 – H3K27 methylation and polycomb (PcG) mediated gene silencing. EZH2 of PRC2 complex di- or tri- methylates H3K27 residues, while EED and SUZ12 have polycomb stabilising properties. Once methylated, H3K27 residues are recognised by the chromodomains of CBX proteins in the PRC1 complex. BMI1 stimulates the RING1 ubiquitin ligase which mono-ubiquitinates H2AK119 residues. On the other hand, polyhomeotic (PH) proteins are responsible for the dimerising of PRC1 complexes and thereby inducing a moderate level chromatin compaction.

1.3.3.4 H3K9 methylation and heterochromatin protein 1

Being regarded as the 'hallmark' of heterochromatin, methylation of H3K9 residues is classically associated with the HP1 mediated repression of transcription. From *Drosophila* to human, H3K9 methylation is predominantly enriched in silenced genomic regions, particularly at constitutive heterochromatin as in the case of pericentromeric or telomeric repeats (review-Jenuwein and Allis, 2001; review-Lee *et al.*, 2005b; Peters *et al.*, 2001). On the other hand, recent genome wide ChIP-sequencing experiments performed on human and mouse cells revealed that the functional consequences of H3K9 methylation could be more complex than previously described. It is very intriguing to note these studies detected H3K9 methylation on transcriptionally active genes as well (Barski *et al.*, 2007; Mikkelsen *et al.*, 2007; Vakoc *et al.*, 2006; Wang *et al.*, 2008). Specifically, poorly investigated mono-methylated forms were reported to be enriched for active genes, whereas extensively studied di- and tri- methylated forms are more pronounced at the promoters and coding regions of genes within facultative and constitutive heterochromatin respectively. It is noteworthy; however, that di- and tri- methylated H3K9s also appear on transcriptionally active genes to a relatively low extent and mainly at the coding regions. The exact reason for and characterisation of this specific distribution pattern has not yet been clarified, but one may speculate that different degrees of H3K9 methylation could serve as a regulatory switch and thereby allow rapid changes in gene expression in response to stimuli. So far, the overwhelming numbers of publications have addressed the repressive effects of H3K9 methylation.

Several H3K9 specific HKMTs were identified for the methylation of H3K9 residues. These were found at different compartments of chromatin and mainly associated with different levels of gene silencing. SUV39H (*Drosophila*, suppressor of variegation 3-9 homologue; two isoforms -1 and -2) was the very first identified HKMT and was found to catalyse H3K9 tri-methylation in mammals (Rea *et al.*, 2000). The activity of SUV39H is predominantly associated with constitutive heterochromatin (Lachner *et al.*, 2001; Peters *et al.*, 2001). There are a few cases where SUV39H was reported to participate in the silencing of euchromatic genes as well. One important example is the recruitment of SUV39H to the *Cyclin-e* promoter due to its interaction with the transcription repressing factor retinoblastoma protein (RB) in mammalian cells (Nielsen *et al.*, 2001). Another important HKMT in this group is G9a, which catalyses mono- and di-methylation of H3K9 residues of euchromatic genes (Rice *et al.*, 2003; Tachibana *et al.*, 2001; Tachibana *et al.*, 2002). For instance, G9a was shown to be recruited to the promoters of many neuronal genes *in vivo* via its interaction with NRSF/REST repressor complex, which represses neuron specific genes outside the nervous system (Roopra *et al.*, 2004). The activity of G9a was related with

another HKMT GLP/EuHMTase which is also able to catalyse mono- and di- methylation of H3K9 residues (Ogawa *et al.*, 2002). Their combinatorial activity was shown to regulate euchromatic genes in relation to murine embryogenesis (Tachibana *et al.*, 2005). SETDB1 (SET domain bifurcated 1, also known as “ESET”) is another H3K9 specific methyltransferase, which can catalyse the transferring of di- or tri-methyl groups and is mainly associated with euchromatic gene silencing (Schultz *et al.*, 2002; Yang *et al.*, 2002). SETDB1 was shown to be recruited to the silenced promoters of endogenous oncogenes such as *P53BP2* and *RASSF1a* in human cancer cells (Li *et al.*, 2006c). Finally, a previously described tumour suppressor PRDM2 (PR domain containing 2, also known as “RIZ1”) was also reported to di-methylate euchromatic H3K9 residues at some murine oncogene promoters such as *c-Myc* and estrogen responsive genes in mice (Carling *et al.*, 2004; Gazzero *et al.*, 2006; Kim *et al.*, 2003).

Demethylation of H3K9 residues is primarily performed by JmjC-domain HKDMs. JHDM2A was shown to specifically demethylate mono- and di- methylated forms of H3K9 residues, whereas JHDM3A, JMJD2A and JMJD2C can act on tri-methylated H3K9s (Cloos *et al.*, 2006; Fodor *et al.*, 2006; Klose *et al.*, 2006; Tsukada *et al.*, 2006; Whetstine *et al.*, 2006; Yamane *et al.*, 2006). These enzymes were associated with transcriptional activation of silenced genes. For example, JHDM2A is recruited to the promoters of androgen-receptor target genes in the presence of androgen hormone in human cells (Yamane *et al.*, 2006). It is also noteworthy that the H3K4 specific LSD1 was also shown to demethylate H3K9 residues, when coupled with the androgen receptor (AR) (Metzger *et al.*, 2005). Another example is the recruitment of JMJD2C to the promoter of oncogenes such as *Mdm2* in mouse embryonic fibroblasts (MEF) (Ishimura *et al.*, 2009).

The effect of H3K9 methylation on chromatin is tightly linked to its functional partner heterochromatin protein 1 (HP1), therefore one should understand the character of this key protein in order to gain a better insight into the impact of H3K9 methylation on chromatin function. HP1 exists in three isoforms in mammals: HP1 - α , - β and - γ . In terms of structure, HP1 proteins consist of an N-terminal chromodomain (CD), a hinge domain and a C-terminal chromoshadow domain (CSD). Each domain provides a different mechanistic link between HP1 and chromatin [Figure 1.10A]: Firstly, the CD domain recognises methylated H3K9 residues as a binding platform (Bannister *et al.*, 2001; Lachner *et al.*, 2001). This recognition was proven to be essential for the establishment of HP1 mediated heterochromatin since knocking out H3K9 specific HKMTs or increasing the acetylation levels of these key residues with HDAC inhibitors triggered the displacement of HP1 in mammalian cells (Cheutin *et al.*, 2003; Taddei *et al.*, 2001). Notably, HP1 recognises all levels of H3K9 methylation but its

binding efficiency is lower for –mono and –di methylated forms *in vitro* (Fischle *et al.*, 2003). This implies different levels of HP1 effects with respect to the degree of H3K9 methylation. Once bound to chromatin, HP1 proteins are able to propagate a high level chromatin compaction facilitated by their CSD domains, which allow their dimerization [Figure 1.10B] (Brasher *et al.*, 2000; Nakayama *et al.*, 2001). The CSD domain was also shown to interact with HKMTs and other chromatin related proteins such as DNA methyltransferases (DNMTs) or HDACs and this is thought to be important for heterochromatin stabilisation or its spreading to neighbouring regions (review-Hiragami and Festenstein, 2005; review-Kwon and Workman, 2011). Proteins that directly interact with HP1 contain a PxVxL penta-peptide motif (Brasher *et al.*, 2000; Cowieson *et al.*, 2000). Amongst these interactions, perhaps the most exciting one is with SUV39H since it seems essential for stimulating the spreading of heterochromatin (Aagaard *et al.*, 1999; Bannister *et al.*, 2001; Schotta *et al.*, 2002). Lastly, the hinge domain that resides between CD and CSD domains was reported to interact with DNA, RNA and histone H1, further strengthening the link between HP1 and chromatin (Maison *et al.*, 2002; Meehan *et al.*, 2003; Muchardt *et al.*, 2002). HP1 isoforms were also reported to interact with SUV4-20H1 which is a heterochromatin specific HKMT that acts on H4K20 residues (Schotta *et al.*, 2004). This interaction seems to require the full length of HP1 proteins and provides a synergistic link between the methylation of H3K9 and H4K20 residues during the establishment of heterochromatin (Hines *et al.*, 2009). Moreover, phosphorylation of the H3S10 residue, which is adjacent to H3K9, releases HP1 proteins bound to methylated H3K9s. This is known as the “methyl-phospho binary switch” and was found to be essential for the delocalisation of HP1 during mitosis (Fischle *et al.*, 2005; Hirota *et al.*, 2005).

It is also noteworthy that all mammalian HP1 isoforms are also extensively decorated with HP1 post-translational modifications including phosphorylation, methylation, acetylation, sumoylation and ubiquitination (LeRoy *et al.*, 2009). These modifications are likely to introduce significant structural changes and assign distinct roles for HP1. Amongst these, phosphorylation has been the most studied one so far. For example, phosphorylation of the HP1 γ isoform at serine-83 is associated with its euchromatic localisation in mammalian cells (Lomberg *et al.*, 2006). Moreover, phosphorylation of HP1 β at threonine-51 was associated with its mobilisation at DNA damage repair sites in live mouse embryonic fibroblasts (Ayoub *et al.*, 2008). Lastly, a recent study reported that sumoylation of HP1 α in the hinge domain is needed for its deposition into major satellite repeats at the mouse pericentromeric heterochromatin (Maison *et al.*, 2011). Although these few studies provide good examples, post-translational modifications of HP1 should be studied to a further extent because of their high regulatory potential in the HP1-mediated control of gene expression. HP1 isoforms are

found in various chromatin compartments and exert a number of important functions via their interaction with methylated H3K9 residues (review-Hiragami and Festenstein, 2005; review-Kwon and Workman, 2011). Generally speaking, HP1 α and β are often detected at heterochromatic regions, whereas HP1 γ is found at euchromatic locations as well (Minc *et al.*, 1999; Minc *et al.*, 2000). Being a classic example of constitutive heterochromatin, centromeres were shown to be decorated with HP1 α and β in mammals (Furuta *et al.*, 1997; Minc *et al.*, 1999). Studies in *Drosophila* and fission yeast *Saccharomyces pombe* revealed that centromeric HP1s facilitate successful loading of cohesins and thereby maintain efficient chromosomal segregation (Kellum and Alberts, 1995; Nonaka *et al.*, 2002). In addition, mammalian and *Drosophila* HP1 isoforms were detected in telomeric heterochromatin, where they are required for telomere capping, elongation and transcriptional repression of these sequences (Garcia-Cao *et al.*, 2004; Perrini *et al.*, 2004). Interestingly, *in vitro* and *ex vivo* experiments demonstrated the interaction of all mammalian HP1 isoforms with various components of the nuclear lamina (Kourmouli *et al.*, 2000; Polioudaki *et al.*, 2001). This could explain the observation that centromeric repeats and repressed heterochromatic genes are typically localised close to the nuclear periphery, where they do not have sufficient access to transcriptional factors and machineries (Alcobia *et al.*, 2000; Andrulis *et al.*, 1998; Dietzel *et al.*, 2004; Hiragami-Hamada *et al.*, 2009).

The repressive function of HP1 within euchromatin was shown to be facilitated by its direct recruitment to the target promoters, where it can form a more restricted and less compact heterochromatin-like structure. This is achieved by the straightforward interaction of HP1 with numerous transcriptional repressors which are usually found in a complex accompanied by H3K9 specific HKMTs and HDACs (review-Hiragami and Festenstein, 2005; review-Kwon and Workman, 2011). For instance, human HP1 γ can interact with E2F-6 repressor complex, which contains H3K9 specific and euchromatic HKMTs; G9a and EuHMTase. This interaction was shown to be crucial for the negative regulation of E2F- and myc-responsive genes such as *E2F1*, *c-MYC* and *CDC25A* (Ogawa *et al.*, 2002). Another important example is the interaction of HP1 isoforms with TIF1 β (transcriptional intermediary factor 1 β , also known as “KAP1”), to form a transcriptional repressor complex (Cammass *et al.*, 2000; Nielsen *et al.*, 1999; Ryan *et al.*, 1999). Notably, TIF1 β also interacts with the euchromatin-specific H3K9 methyltransferase SETDB1 and was found in a complex comprising the transcriptional co-repressors N-CoR and NuRD (Schultz *et al.*, 2002; Schultz *et al.*, 2001; Underhill *et al.*, 2000). Interaction of TIF1 β and HP1 was reported to be essential for mouse endodermal differentiation in mice by regulating the expression of key genes (Cammass *et al.*, 2002).

As opposed to the repressing roles described above, few studies suggest a transcriptionally positive role for HP1 proteins. This effect seems to be specific mostly for the γ -isoform, which was shown to interact with the FACT complex and elongating RNAPII revealed both by *in vitro* assays and mammalian *ex vivo* systems (Kwon *et al.*, 2010; Vakoc *et al.*, 2005). It has been proposed that HP1 γ in this case might facilitate stabilisation of nascent transcript and RNAPII associated factors (review-Kwon and Workman, 2011). Several studies also suggest transcriptionally negative roles for HP1 γ at the gene promoters. One example was described above (i.e. E2F and myc responsive genes) and silencing of the HIV (human immunodeficiency virus) long terminal repeat (LTR) in post-integration latency could be given as another important case (du Chene *et al.*, 2007). Overall, one may consider a transcription inactivating role for HP1 γ at repressed euchromatic gene promoters and a positive role for transcriptional elongation along the coding regions of actively transcribed genes. This fits well with the above-described distribution pattern of methylated H3K9 residues along coding regions of actively transcribed genes. Lastly, a co-localisation study performed on mouse embryonic fibroblasts also suggested a transcription activating function of HP1 β in the RNAPI mediated transcription of rDNA genes (Horakova *et al.*, 2010). Although these intriguing results opened a new avenue in the gene regulation field, there is still much further to go in order to address the precise function and mechanism of these recently described phenomena. However, the post-translational modifications described above could provide an explanation for 'distinct' and perhaps 'opposite' roles for HP1 within different contexts.

HP1 proteins were also studied within the frame of DNA replication and repair. Mammalian HP1 isoforms were reported to interact with CAF1 and this was shown to be crucial for the stability and inheritance of heterochromatin during replication (Murzina *et al.*, 1999; Quivy *et al.*, 2008). A similar function was also reported both in *Drosophila* and mouse due to the interaction of HP1 proteins with the origin recognition complex 1 (ORC1) (Badugu *et al.*, 2003; Pak *et al.*, 1997; Prasanth *et al.*, 2010). Furthermore, another dimension of HP1 function seems to involve DNA damage response. According to observations in human cells, all HP1 isoforms seem to be recruited to DNA lesions induced by ultraviolet (UV) beams (Goodarzi *et al.*, 2008; Luijsterburg *et al.*, 2009; Zarebski *et al.*, 2009). The exact reasoning and mechanism behind this recently described effect are yet to be determined. One explanation might be rapid transcriptional silencing of damaged regions as a protective cellular response (review-Kwon and Workman, 2011). Although, DNA damage related placement of HP1 was reported to be H3K9 methylation independent, several studies suggested H3K9me3 as an initial response for double-strand breaks (Peng and Karpen, 2009; Sun *et al.*, 2009).

Figure 1.10

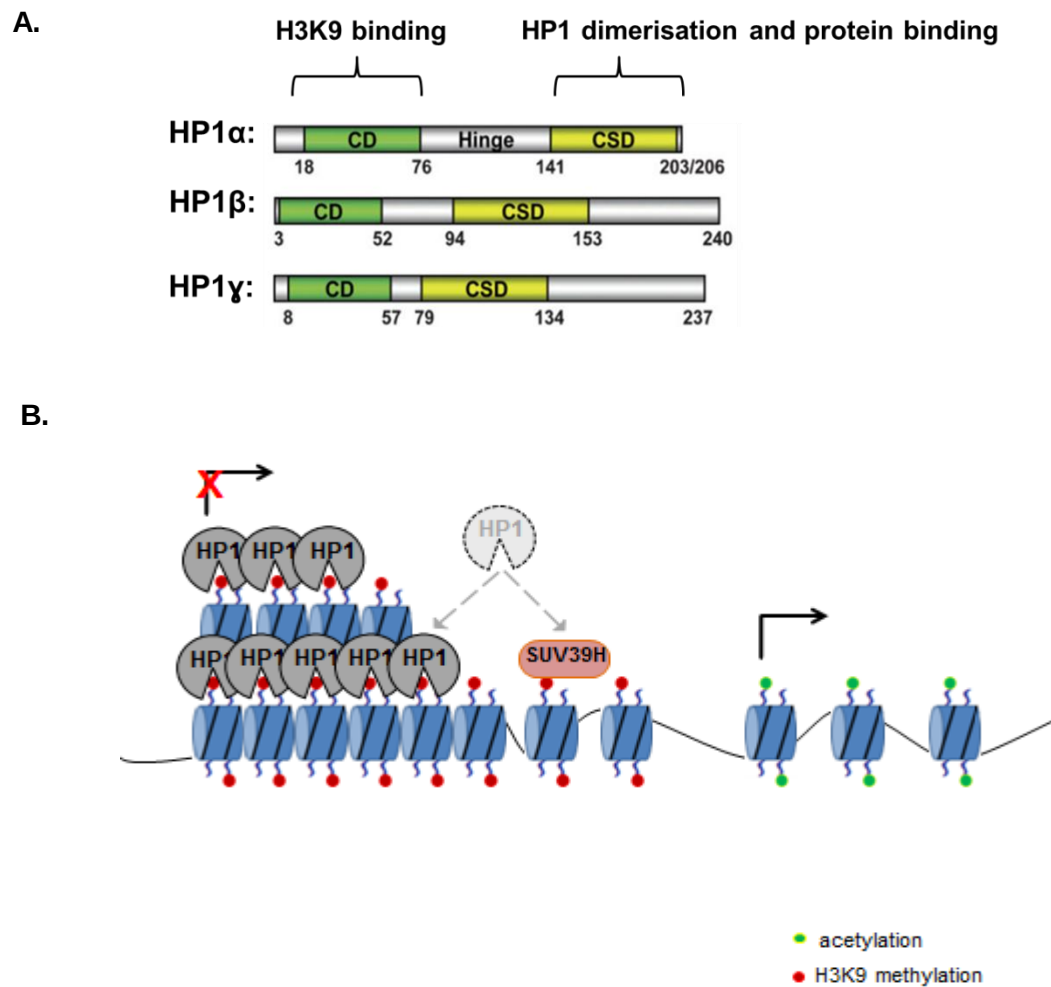


Figure 1.10 – HP1 isoforms and heterochromatinisation by HP1. [A] **HP1 isoforms.** HP1 has three isoforms which contain a H3K9 binding chromodomain (CD), a linker hinge domain and a chromoshadow domain (CSD) that is involved in HP1 dimerisation and binding to other proteins. [B] **Heterochromatinisation by HP1.** SUV39H tri-methylates H3K9 residues and this is recognised by the HP1 proteins resulting in a high level of chromatin compaction. Interestingly, SUV39H interacts with the CSD domain of HP1. This interaction and self-dimerising feature of HP1 proteins facilitates the propagation of heterochromatin. [A is adapted from (review-Kwon and Workman, 2011)]

1.4 Other mechanisms of epigenetic gene regulation

1.4.1 DNA methylation

DNA can be covalently modified at cytosine residues of 5'CpG3' dinucleotide sequences by DNA methyltransferases DNMT -1, -3a/b (Bestor and Ingram, 1983; Holliday and Pugh, 1975; Li *et al.*, 1992; Riggs, 1975). DNMTs act at various contexts during the embryonic development in order to establish specific methylation patterns (review-Geiman and Muegge, 2010). Although this process seems to be reversible, no DNA specific demethylases have been identified so far (Ramchandani *et al.*, 1999). 60% of human promoters contain CpG islands (Shen *et al.*, 2007), which can regulate transcription either by disrupting proteins that bind to unmethylated CpGs (e.g. CTCF), or attracting methylated CpG binding proteins (e.g. MeCP2) (review-Dhasarathy and Wade, 2008). These are known to recruit specific chromatin factors (e.g. MeCP2 complexes recruit HDACs). Moreover, DNMTs are known to associate with repressive chromatin factors (e.g. SUV39H & HP1 and DNMT3a/b) (review-Cedar and Bergman, 2009). Notably, dysregulation of DNA methylation was linked to diseases including cancer and neurodegenerative disorders (review-Robertson, 2005).

1.4.2 RNA interference mediated gene silencing

RNA interference (RNAi) is an evolutionarily conserved phenomenon, which is based on post-transcriptional silencing of genes through small interfering RNAs (siRNA) (Caplen *et al.*, 2001; Elbashir *et al.*, 2001; Fire *et al.*, 1998). Endoribonuclease activity of Dicer enzyme generates siRNAs from double stranded RNA (dsRNA), which can be formed either by secondary RNA structures (e.g. small hairpin RNA, microRNA) or antisense transcription activity. These siRNAs are subsequently incorporated into the RNA-induced silencing complex (RISC), which cleaves mRNAs in a sequence specific manner. The signal can then be amplified by the activity of RNA dependent RNA polymerases (RDRP), which generate further siRNAs (review-Mello and Conte, 2004). In *S. pombe*, an additional mechanism was defined at the level of chromatin. This is particularly obvious at centromeric repeats, where dsRNAs occurring as a result of repeat transcription is recognised and cleaved by the RNA-induced transcriptional gene silencing (RITS) complex. RITS recruits CLR4 (SUV39H homologue) and causes subsequent H3K9 methylation (review-Djupedal and Ekwall, 2009; Reinhart and Bartel, 2002; Volpe *et al.*, 2002). There are contradictory reports which address the conservation of this mechanism in mammals and this still remains unresolved (Bernstein *et al.*, 2003; Fukagawa *et al.*, 2004; Murchison *et al.*, 2005; Wang *et al.*, 2006).

1.5 Lessons from position effect variegation and control mechanisms against the spreading of heterochromatin

1.5.1 Position effect variegation

The strong connection between chromatin structure and gene activity largely originated from early observations on fruit fly (*Drosophila melanogaster*) where the expression of a euchromatic gene called “white” is responsible for red eye colour. In 1930, Hermann Muller described a radiation-induced translocation of the *white* gene into close proximity of centromeric heterochromatin and observed its stochastic inactivation that results in a mosaic pattern on eye colour [Figure 1.11] . This phenomenon is known as position effect variegation (PEV) and was seen in various organisms ranging from yeast to mouse when a reporter gene is placed close to centromeric, pericentromeric or telomeric heterochromatin [see section 1.8.3 and figure 1.21 for further information] (Allshire *et al.*, 1994; Baur *et al.*, 2001; Festenstein *et al.*, 1996; Gottschling *et al.*, 1990). Stochastic expression profiles of PEV-exhibiting genes among cell populations were shown to be stable over many generations. Therefore, the decision whether to express or repress a gene subject to PEV is thought to be taken early during the development of a specific cell lineage (Alami *et al.*, 2000; Grewal and Klar, 1996; review-Kioussis and Festenstein, 1997; review-Singh, 1994). Stably inherited patterns of PEV imply an important role for epigenetic mechanisms during this process (review-Dillon and Festenstein, 2002; review-Festenstein and Kioussis, 2000; review-Kioussis and Festenstein, 1997). Consequently, PEV provided an excellent model system for conducting research on epigenetic gene regulation (review-Li and Sasaki, 2011; review-Probst *et al.*, 2009). Indeed, many chromatin regulating proteins were elucidated using this phenomenon as an experimental approach. Perhaps the most striking examples are heterochromatin related HKMT SUV39H and its partner HP1, which were initially identified as modifiers of PEV in *Drosophila* genetic screens (James and Elgin, 1986; Rea *et al.*, 2000; review-Reuter and Spierer, 1992; Tschiersch *et al.*, 1994).

Figure 1.11

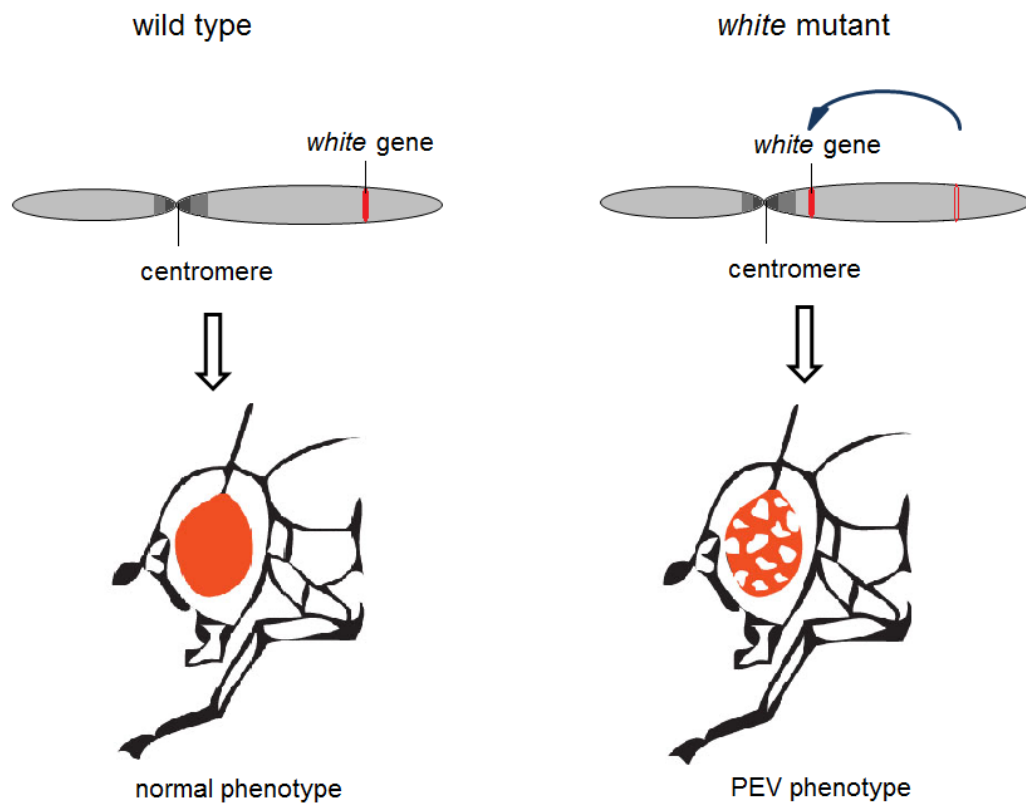


Figure 1.11 - Archaic PEV model on *Drosophila* eye colour. The *white* gene is responsible for the red eye colour on *Drosophila* and is normally located on a euchromatic chromosomal region. However, radiation-induced translocation of this gene to a region close to pericentromeric heterochromatin causes its repression in a proportion of single eye cells. The result is a mosaic eye colour formed by white and red patches of eye cells.

1.5.2 Competition between euchromatin and heterochromatin

Most PEV modifiers were found to have a dosage-dependent effect, leading to a hypothetical 'mass-action' model for PEV (review-Dillon and Festenstein, 2002; Festenstein *et al.*, 1999; Locke *et al.*, 1988; Tartof *et al.*, 1989; review-Zuckerandl, 1974). Accordingly, relative abundance and local concentration of individual components involved in the formation of heterochromatin (e.g. HKMTs, HATs, HDACs, HP1, PcG etc.) are primary rate limiting factors for the degree of PEV. Furthermore, the balance of these factors with their counteracting factors (e.g. HAT vs HDAC) is crucial for regulating the extent of PEV. Another important layer is the presence of regulatory elements such as enhancer, activator, silencer or insulator elements and locus control regions (LCRs), as well as antisense transcription and DNA methylation [Figure 1.12]. Secondly, the number of sites targeted by chromatin proteins appears to be a PEV-limiting parameter since introducing an extra heterochromatic Y chromosome suppresses variegation in *Drosophila* (Dimitri and Pisano, 1989). In this case, presence of an increased amount of heterochromatinised DNA within the nucleus is thought to titrate away silencing chromatin factors from other heterochromatic regions, providing a Y chromosome 'sink' effect and thereby inhibiting heterochromatin-mediated gene silencing in other genomic regions. By analogy with this observation, one may also expect the inactivated X chromosome in mammalian females to act like a 'sink' for heterochromatin factors (Chadwick and Willard, 2003b). These observations based on PEV-models suggest competition between euchromatic and heterochromatic factors (review-Dillon and Festenstein, 2002). The probability of a gene being active or inactive should be a result of the final outcome of this competition.

Figure 1.12

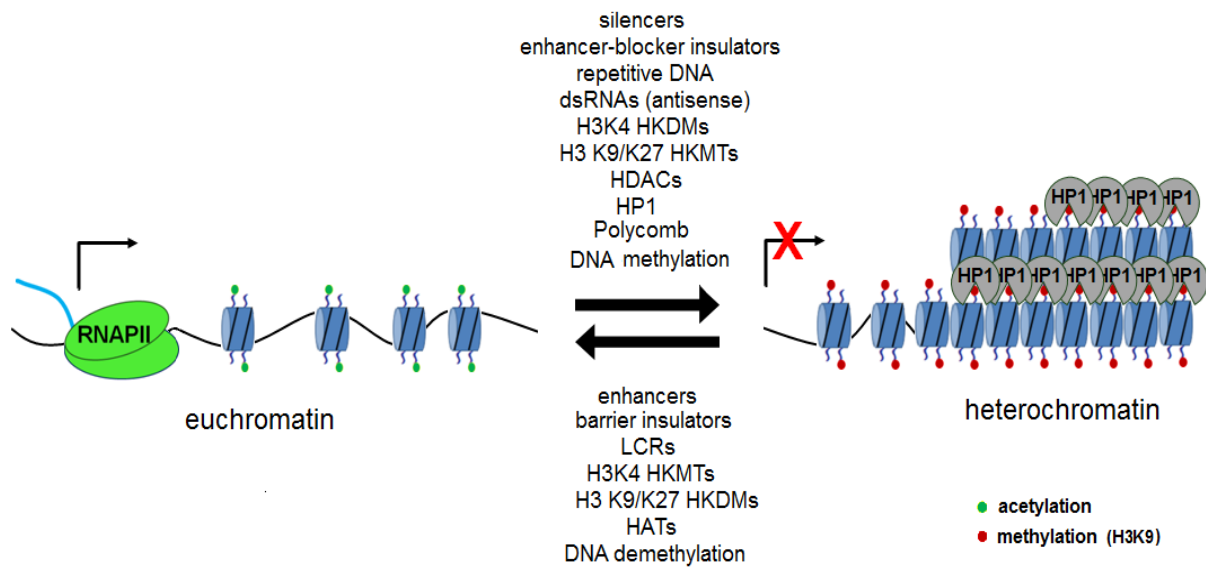


Figure 1.12 - Competition between euchromatin and heterochromatin. The fact that PEV exhibits a dosage dependent effect for individual components of heterochromatin leads to the idea that local concentrations of each factor could greatly influence the expression state of a given gene, known as “mass-action effect”. In a eukaryotic nucleus, heterochromatic factors such as HP1 and H3K9 specific HKMTs are in competition with euchromatic factors such as H3K9 specific HKDMs and HATs. Various combinations of these factors could increase the probability of a gene being euchromatic or heterochromatic. In rare cases, the balance of competing factors could be in equilibrium causing stochastic gene expression (PEV).

1.5.3 Spreading of heterochromatin

Studies in *Drosophila* revealed that the PEV effect could take place even if the variegating gene is located several megabases away from heterochromatin and this led to the hypotheses of *cis*-spreading or *trans*-interacting heterochromatin (Demerec and Sutton, 1940; Hayashi *et al.*, 1990; Tartof *et al.*, 1984; review-Weiler and Wakimoto, 1995). According to the *cis*-spreading model, heterochromatin propagates in a linear fashion into neighbouring regions. This explanation fits well with the self-dimerising nature of heterochromatin proteins and their ability to recruit partner chromatin modifiers (e.g. SUV39-H and HP1) providing a positive feedback for heterochromatin formation and its spreading. Muller's original experiment with the *Drosophila* eye colour is a well-known example for *cis*-spreading. The *trans*-interacting model; on the other hand, considers the overall three-dimensional chromatin organisation and the possibility of interactions from non-proximal locations. A typical example for this model is the silencing of the *Drosophila* brown eye colour gene due to a long distance association with pericentromeric heterochromatin as a result of its homologous pairing with its mutant heterochromatic allele (Csink and Henikoff, 1996; Demerec and Sutton, 1940).

1.5.4 Chromatin insulators

In order to deal with the intruding effects of heterochromatin or blocking inappropriate interactions between adjacent regulatory sequences cells have evolved chromatin insulator elements. There are two types of function reported for chromatin insulator proteins which recognise specific DNA sequences. Enhancer-blocking insulators can perturb activation of a gene primarily by creating loops via intramolecular interactions and thereby physically disrupting the communication between enhancer and promoter [Figure 1.13A] (review-Gaszner and Felsenfeld, 2006). These elements may also obstruct a processive RNAPII or compete with *bona fide* promoters via attracting transcriptional activators (review-Bushey *et al.*, 2008; review-Geyer, 1997; Kong *et al.*, 1997). Importantly, some insulators may act as heterochromatin barriers and prevent the propagation of heterochromatin into certain regions where active transcription is taking place [Figure 1.13B]. This could be achieved by their ability to recruit euchromatin-promoting factors (e.g. HATs, H3K4 specific HKMTs etc.), tethering chromatin to a euchromatin-rich subnuclear environment or breaking continuous arrays of nucleosomes (review-Bi and Broach, 2001; review-Gaszner and Felsenfeld, 2006; Oki and Kamakaka, 2005).

A well-studied model for chromatin insulation is the chicken β -globin locus, where two insulator elements with enhancer-blocking and heterochromatin barrier activities were found

(review-Bushey *et al.*, 2008; Chung *et al.*, 1997; Chung *et al.*, 1993). Here, enhancer blockage is facilitated primarily by the prototype insulator protein CTCF (CCCTC-binding factor) whereas heterochromatin barrier function is reportedly performed by upstream transcription factors USF1 and USF2 (Bell *et al.*, 1999; West *et al.*, 2004a). In mammals, the same locus was shown to be regulated in a similar fashion and exhibit long-range loopings formed by CTCF-CTCF interactions (Splinter *et al.*, 2006). CTCF can also demarcate heterochromatin boundaries primarily within the context of H3K27 methylation and reduce chances for transcriptionally-repressive nuclear lamina interactions (Barski *et al.*, 2007; Bartkuhn *et al.*, 2009; Cuddapah *et al.*, 2009; Guelen *et al.*, 2008). Furthermore, many active promoters in mammalian cells were shown to be occupied by CTCF, where it directly interacts with RNAPII (Barski *et al.*, 2007; Bartkuhn *et al.*, 2009; Chernukhin *et al.*, 2007). In line with these observations, several tumour suppressor genes (i.e. *P16*, *CHD1*, *RASSF1A*) were found to be regulated by CTCF, which is thought to recruit euchromatic enzymes on gene promoters and thereby prevent the spreading of adjacent heterochromatin (Witcher and Emerson, 2009). However, whether CTCF is responsible for the direct recruitment of certain chromatin factors, is not yet clear. Finally, CTCF binding was also suggested to be crucial for X-inactivation centre formation in females (Donohoe *et al.*, 2009; Xu *et al.*, 2007a).

Interestingly, co-localisation of CTCF with cohesins was reported at the imprinted *H19/IGF2* (*H19* non-coding RNA; *IGF2* insulin like growth factor 2) locus in mammalian cells (Wendt *et al.*, 2008). Moreover, cohesin recruitment with or without CTCF seems to play an essential role for effective V(D)J recombination and T-cell receptor rearrangement during mammalian immune system development (Degner *et al.*, 2009; Seitan *et al.*, 2011). These findings, as well as previous studies in yeast and *Drosophila* suggest enhancer-blocking as well as barrier insulator functions for cohesin, which was originally identified as a ring-shaped protein complex holding sister chromatids together enabling efficient segregation during cell division (Gruber *et al.*, 2003; Michaelis *et al.*, 1997; review-Peric-Hupkes and van Steensel, 2008). RNAi knockdown experiments revealed that SCC1/RAD21 or SMC3 subunits of cohesin are essential for context-dependent CTCF insulation activity (Haering *et al.*, 2008; review-Peric-Hupkes and van Steensel, 2008). Although the exact functional implications of the CTCF-cohesin cooperation is not yet clear, their similar genomic distribution patterns suggest a tight association between these two factors (Parelho *et al.*, 2008; Rubio *et al.*, 2008). It is thought that cohesin's ability to encircle DNA fibres *in trans* provides another layer on top of CTCF-mediated loopings, helping to block unwanted regulatory interactions or recruiting the bound gene into a different chromatin environment (Haering *et al.*, 2008; review-Peric-Hupkes and van Steensel, 2008).

Figure 1.13

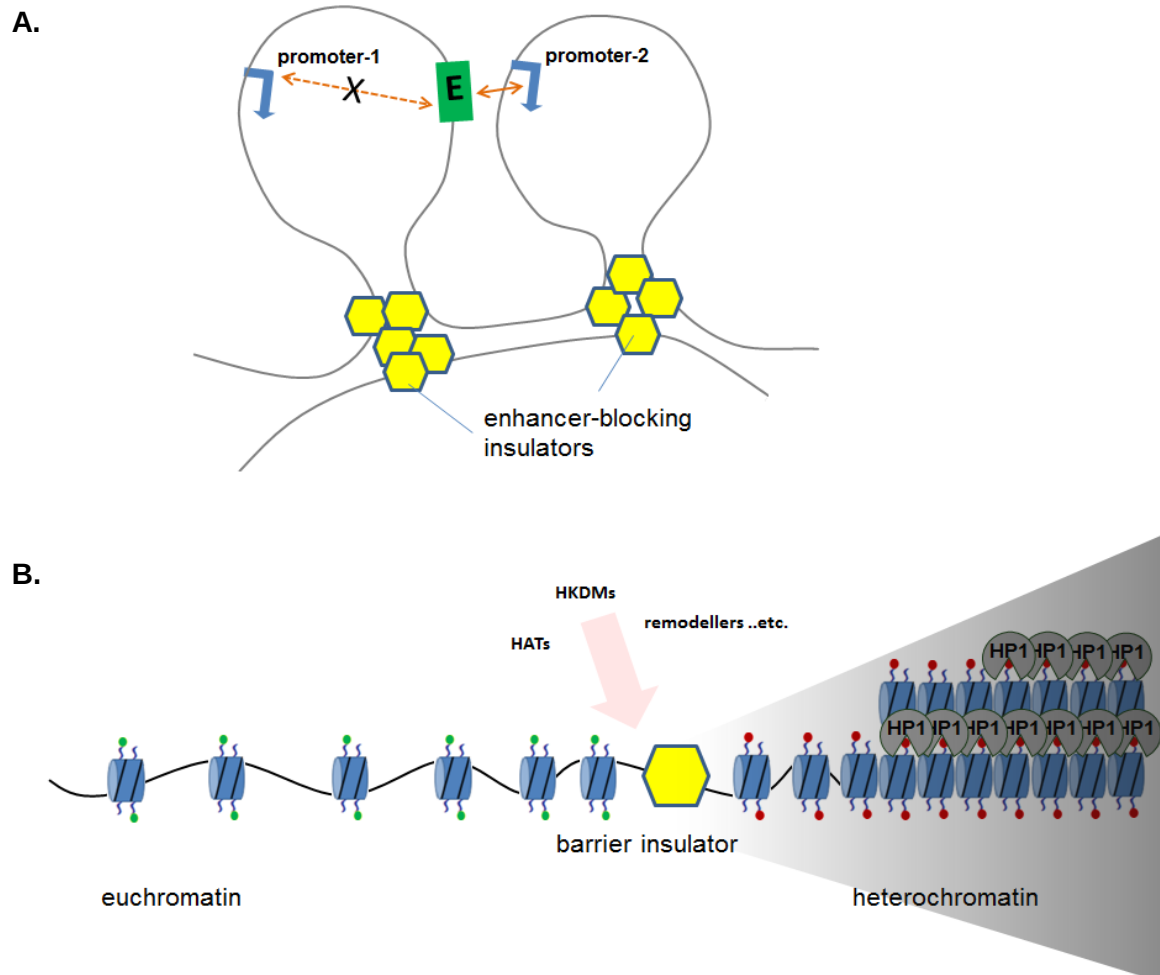


Figure 1.13 - Chromatin insulation mechanisms. **[A] Enhancer-blocking insulation.** These elements function by forming DNA loops which are mediated by intramolecular insulator interactions. As seen in the regulation of *β -globin* locus, such physical conformations of DNA may obstruct enhancer communication with one promoter while it favours interaction with another. **[B] Heterochromatin-barrier insulation.** As can be seen in the regulation of various oncogenes (i.e *P16*, *CHD1*, *RASSF1A*) some insulator elements can stop heterochromatin invasion into an actively transcribed gene via direct recruitment of euchromatic factors (HATs, HKDMs etc.), tethering chromatin to a euchromatic subnuclear environment or physically breaking continuous arrays of nucleosomes.

1.5.5 Locus control regions (LCRs)

Another regulatory element that can overcome heterochromatin invasion and regulate gene expression is the locus control region (LCR), which was initially identified during the development of transgenic mouse technology. In early attempts, transgenes would typically exhibit a PEV-like silencing when randomly integrated close to a heterochromatic region (review-Kioussis and Festenstein, 1997). This problem was overcome by the identification of LCRs (Forrester *et al.*, 1987; Forrester *et al.*, 1986; Grosveld *et al.*, 1987). These are *cis*-acting regulatory elements characterised by a number of DNase-I hypersensitive sites (HSSs) that exhibit regulatory activities similar to enhancers and insulators (review-Li *et al.*, 2002). LCRs are typically located ~6-60kb away from the 5' or 3' end of a gene cluster and their significant impact on each gene within the cluster could be explained by the long-range interactions of LCRs with individual gene promoters (review-Kioussis and Festenstein, 1997; review-Miele and Dekker, 2008). During development, LCRs were shown to facilitate tissue-specific gene expression independently of genomic position (review-Kioussis and Festenstein, 1997).

The classical example for LCR function is the regulation of the mammalian β -globin cluster, which contains several genes arranged in a developmentally regulated tandem (review-Mahajan *et al.*, 2007). Mammalian β -globin LCR consists of six HSSs (HSS 1-6 in 3' to 5' order) which are sequentially activated during erythrocyte development. HS1-4 were shown to perform enhancer activities and can directly interact with the promoters of expressed β -globin genes as shown by DNA-TRAP (tagging and recovery of associated proteins) and 3C (chromosome conformation capture) techniques (Carter *et al.*, 2002; Casolari *et al.*, 2004; Palstra *et al.*, 2003; Tolhuis *et al.*, 2002). Interestingly, regions actively interacting with HSSs were shown to be enriched by the transcription factors NF-E2 and GATA1, as well as active histone marks such as H3/H4 acetylation and H3K4 methylation (Bulger *et al.*, 2003; Forsberg *et al.*, 2000; Johnson *et al.*, 2001; Letting *et al.*, 2003). On the other hand, the HSS5 of the mammalian β -globin LCR was shown to possess CTCF-mediated insulation activity (Tanimoto *et al.*, 2003). Importantly, deletion mutations within the LCR region were shown to cause γ - β -thalassemia (Kioussis *et al.*, 1983). In line with this fact, transgenic mice with full LCR showed position-independent and transgene copy number-dependent expression of the β -globin gene (Grosveld *et al.*, 1987; Milot *et al.*, 1996), whereas mice with disrupted or no LCR conversely exhibited very low level and position-dependent expression (Kollias *et al.*, 1986; Magram *et al.*, 1989; Milot *et al.*, 1996). Apart from β -globin, more than 20 mammalian gene loci were shown to be regulated by LCRs (review-Li *et al.*, 2002).

1.5.6 hCD2 mammalian PEV model

One of the important examples for LCR function is the human CD2 gene, which encodes the constitutive T-cell specific marker CD2, a 50 kDa glycoprotein facilitating the interaction between T-cells and antigen presenting cells (review-Bierer and Hahn, 1993). A human hCD2 minigene, which consists of the hCD2 cDNA with the first intron (1.5kb) and the flanking 5' (5.6kb) and 3' hCD2 regulatory (~5.5kb) regions, can successfully express human CD2 protein on the surface of mouse thymocytes and T-cells when integrated into the mouse genome (Lang *et al.*, 1988). Its expression was shown to be tightly regulated by a 3' flanking LCR that stimulates transcription in an orientation-independent manner (Greaves *et al.*, 1989; Lake *et al.*, 1990; Lang *et al.*, 1991). This 2kb long LCR is characterised by three hypersensitive sites (HSS) (Festenstein *et al.*, 1996; review-Kioussis and Festenstein, 1997; Lake *et al.*, 1990) HSS1 was shown to exhibit a strong classical enhancer activity whereas HSS2 probably acts as a weak enhancer element. On the other hand, HSS3 has no enhancer activity but is nevertheless necessary for the LCR function (Greaves *et al.*, 1989; Lang *et al.*, 1991; Lang *et al.*, 1988). Notably, deletion of HSS3 caused PEV of hCD2 expression in the transgenic mouse thymus and peripheral T-cells when the hCD2 minigene was integrated into pericentromeric heterochromatin [Figure 1.14] (Festenstein, 1996; Festenstein *et al.*, 1996). Variegation of hCD2 at the protein level was shown to correlate well with hCD2 mRNA levels (Saveliev *et al.*, 2003). One advantage of this system is that it allows one to assess the extent of PEV precisely by using flow cytometry (FACS). Overall, this transgenic model provides an invaluable tool to study PEV and thereby chromatin-mediated gene regulation in mammals. Notably, PEV of the hCD2 transgene was reported to be sensitive to HP1 dosage (a classical *Drosophila* PEV modifier) as double transgenic hCD2 mouse over-expressing HP1 β exhibited more PEV silencing in concordance with what had been observed in *Drosophila* PEV (Festenstein *et al.*, 1999).

Figure 1.14

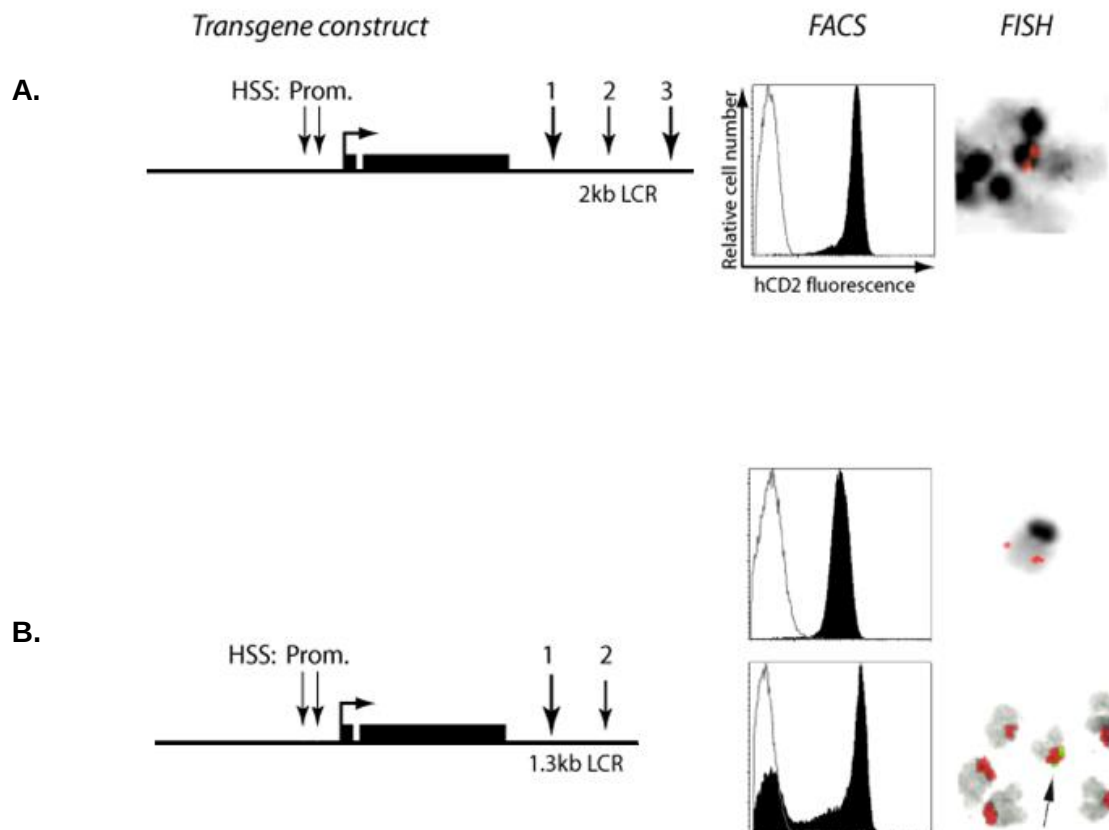


Figure 1.14 - hCD2 transgenic system as a model to study mammalian PEV. [A] **hCD2 expression on T-cells with full-length LCR.** An hCD2 minigene with full LCR (2kb), which has all three HSSs, does not exhibit PEV on hCD2 expression even when it is placed into pericentromeric heterochromatin. [B] **hCD2 expression on T-cells with truncated LCR.** When the third HSS is deleted from the LCR (1.3kb), only a proportion of cells express the transgene as a result of its integration into pericentromeric heterochromatin. [First panel is an illustration for the hCD2 LCR used in the experiments, second panel is the flow cytometry (FACS) analysis and third panel is DNA fluorescence in situ hybridisation (FISH) images where red signal overlaps with major satellites and green with hCD2 transgene. [Adapted from (PhD thesis-Festenstein, 1996; Festenstein *et al.*, 1999; Festenstein *et al.*, 1996)]

1.6 Regulation of RNA polymerase II transcription

1.6.1 RNA polymerase II structure and function

Once the chromatin environment is accessible, a protein coding gene can be transcribed by RNA polymerase II (RNAPII), which also transcribes most non-coding RNAs (Carninci *et al.*, 2005; Roeder and Rutter, 1969). The 'core' RNAPII is a large and evolutionarily conserved multimeric complex (550kDa) that comprises 12 subunits [Figure 1.15] (Cramer *et al.*, 2000; review-Woychik and Hampsey, 2002). Two large proteins, RPB -1 and -2 hold the downstream DNA in jaws as it enters the enzyme. Downstream DNA is held in a bridge until it hits the 'wall' formed by RPB2 at the active site. This clamps DNA and forces it to make a turn towards the exit route. RPB -1 and -2 also form a funnel where the dNTPs enter and a Mg^{2+} containing catalytic site responsible for the nucleotidyltransferase activity. Proper organisation of the RNA-DNA hybrid is ensured by the 'rudder' and 'lid' domains of RPB1. Other subunits which have not been mentioned so far stabilise the overall core RNAPII structure and provide binding sites for regulatory factors (Armache *et al.*, 2003; Bushnell *et al.*, 2002; Bushnell and Kornberg, 2003; Cramer *et al.*, 2001; Gnatt *et al.*, 2001; Westover *et al.*, 2004).

An important part of the core RNAPII is RPB1's carboxy-terminal domain (CTD), which protrudes from the surface of the complex like a 'tail' providing a unique regulatory module. This CTD tail consists of a unique array of tyrosine1-serine2-proline3-threonine4-serine5-proline6-serine7 (YSPTSPS) consensus amino acid repeats, which is present in 52 copies in mammals (review-Corden, 1990; Corden *et al.*, 1985). Although the CTD tail is not required for the RNAPII enzymatic activity *in vitro* it possesses indispensable regulatory functions *in vivo* as its deletion is lethal in mammals, *Drosophila* or yeast (Meininghaus *et al.*, 2000; review-Phatnani and Greenleaf, 2006). CTD function includes recruiting RNA processing or histone modifying factors and providing a switch for various conformational changes in RNAPII (review-Phatnani and Greenleaf, 2006). These functions are substantially regulated by a multitude of reversible post-translational modifications such as phosphorylation, isomerisation and glycosylation (review-Meinhart *et al.*, 2005). Phosphorylation of CTD on serine residues is of particular importance as it plays crucial roles in various stages of transcription (Komarnitsky *et al.*, 2000). Serine-5 (S5) and serine 2 (S2) phosphorylations are specifically associated with transcription initiation and elongation, respectively. On the other hand, S7 phosphorylation has been suggested to perform gene specific functions possibly via affecting the processing of nascent transcripts and influencing the extent of S5 phosphorylation (Chapman *et al.*, 2007). Finally, it is also noteworthy that the CTD tail is ubiquitinated in response to DNA damage (Bregman *et al.*, 1996; Somesh *et al.*, 2005).

Figure 1.15

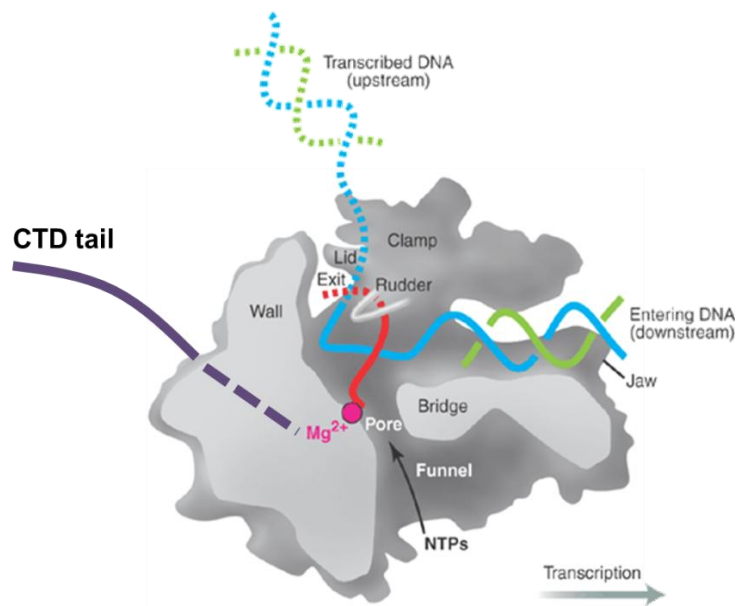


Figure 1.15 - Structure of RNA polymerase II. RNAPII is a multimeric complex comprised of 12 subunits. Downstream DNA enters the complex by protein 'jaws' and is held in a bridge-like conformation until it hits a 'wall' formed by RPB -1 and -2. Here, DNA is forced to make a turn towards the exit channel where it is stabilised by a 'lid' and 'clamp'. As a result, a transcription bubble is formed. RNA is synthesised at the Mg²⁺ containing catalytic site as dNTPs are consumed from the 'funnel'. Throughout the transcription cycle, the carboxyterminal domain (CTD) of the large RPB1 subunit is subject to a number of modifications and provides a centre of attraction for many regulatory factors [Adapted from (Cramer *et al.*, 2000)]

1.6.2 Regulatory factors in association with RNA polymerase II

RNAPII is incapable of transcribing by itself and is thus found in a 'holoenzyme' complex where it interacts with several regulatory factors, which recognise specific sequences on a given eukaryotic gene [Figure 1.16] (review-Hahn, 2004).

General Transcription Factors (GTFs)

GTFs include TFIIB, TFIID, TFIIE, TFIIF and TFIIH, which are essential in terms of promoter recognition and pre-initiation complex (PIC) formation (review-Roeder, 1996). PIC formation takes place at core promoters, which can be constituted by either one or several of the 'core elements'. These include the TATA-box, initiator element (Inr), downstream promoter element (DPE), TFIIB recognition element (BRE) or motif ten element (MTE). In the case of TATA-box containing promoters, TATA-binding protein (TBP) is responsible for recognition whereas DPE and Inr elements are primed by TFIID. BRE elements, on the other hand, are primed by TFIIB (review-Maston *et al.*, 2006). Notably, GTFs are able to facilitate transcription only at a basal level and strong physiological stimulation (and sometimes inhibition) is achieved by the activity of sequence-specific transcription factors *in vivo* (review-Kadonaga, 2004).

Sequence-specific factors

Sequence-specific factors (e.g. P53, OCT1, SP1, SRY etc.) include homeodomain, helix-loop-helix, leucine zipper, zinc finger, nuclear receptor, POU and HMG-motif TFs, which can recognise promoter-proximal regulatory elements, enhancers, silencers or LCRs (review-Narlikar and Hartemink, 2006). Promoter-proximal elements are direction dependent regulatory sequences usually found close to the core promoter. These can bind TFs causing either activation or repression via modulating the affinity for GTFs (review-Lemon and Tjian, 2000). Enhancers and silencers typically reside in promoter-distal regions and act in an orientation-independent manner by binding activating or repressing TFs as well as recruiting relevant chromatin modifiers (review-Dhillon and Kamakaka, 2002; review-Ogbourne and Antalis, 1998). LCR elements also contain strong enhancer activity and have an additional function explained in section 1.5.5.

Regulatory co-factors

Finally, regulatory co-factors (e.g. P300, FACT, N-COR, SIN3 etc.) provide a bridge between other factors and the RNAPII complex or are involved in the recruitment of chromatin modifiers and remodelling machinery (review-Roeder, 2005). TFIIA and TAF components of TFIID and the SRB/mediator complex are well known examples.

Figure 1.16

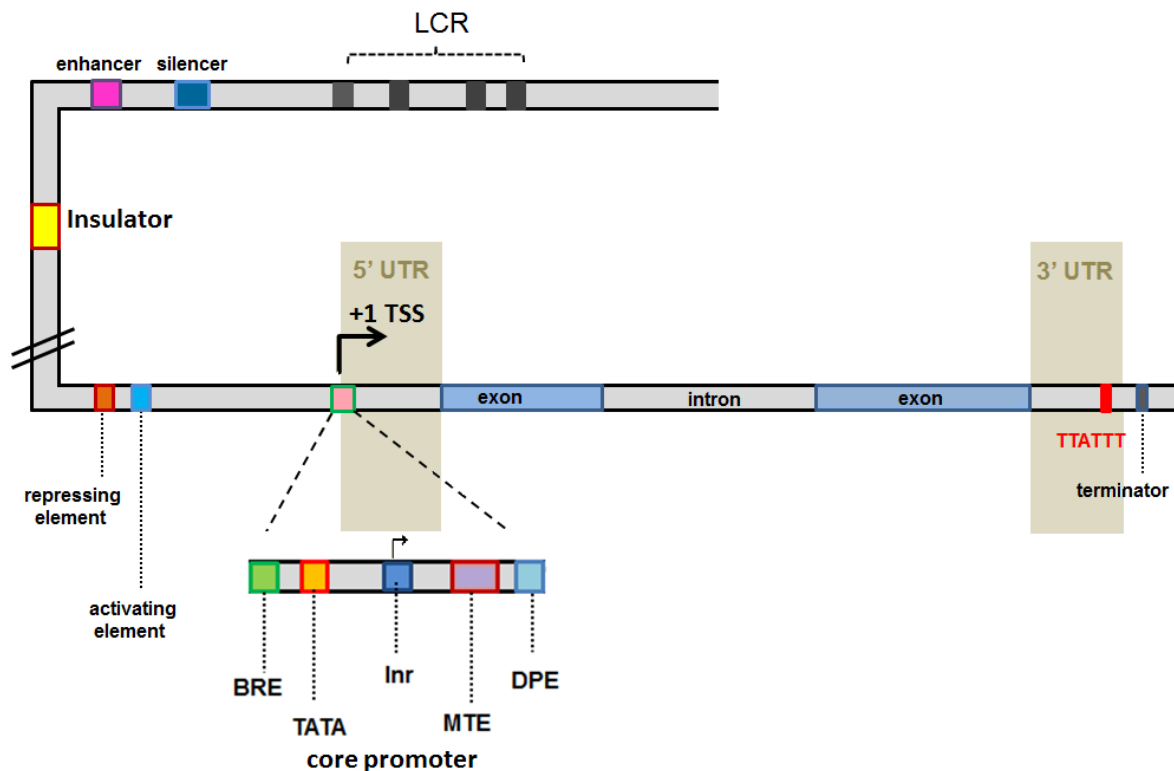


Figure 1.16 - Regulatory elements on a mammalian gene. Transcription is tightly regulated by a number of sequence elements that are recognised by transcription factors. Transcription start site (TSS) is located on a 'core promoter', which consists of one or several main elements (BRE, TATA, Inr, MTE, DPE – see text for definition) that are recognised by general transcription factors and RNAPII. The probability of this recognition is determined by a range of proximal and distal regulatory regions. Repressing and activating elements typically reside close to the core promoter with pivotal implications for the initiation of gene expression at physiological levels. Enhancer and silencer elements, on the other hand, are mostly located a bit further away (although not necessarily) and communicate with the core-promoter or other proximal-elements via specific factors. Locus control regions (LCRs) possess several enhancer-like elements and regulate specific expression patterns during development. Finally, insulators either block the communication between enhancers and promoters or insulate the gene from the neighbouring chromatin effects. RNAPII reads through several exons (protein coding) and introns (subject to splicing), after which it stops at a termination signal (TTATTT) or by terminator DNA sequences in some cases. The resultant transcript has a flanking 5' and 3' untranslated regions (UTR), which promote the stability of mRNA and its efficient translation.

1.6.3 Transcription cycle

Eukaryotic transcription by RNAPII proceeds in a sequence of events which can be categorised in three main phases: initiation, elongation and termination. Notably, current knowledge suggests that elongation can be divided into two sub-stages, where RNAPII pauses firstly and then commits to efficient elongation. In the past most studies in relation to gene regulation addressed initiation as the rate limiting step but more recently elongation has been recognised as an important regulatory step. The main events in transcription cycle are summarised in figure 1.17.

1.6.3.1 Initiation

When a sequence-specific activator binds to a promoter-proximal element, it creates an affinity site for GTFs. Firstly, one or several of the core promoter elements are bound by specific subunits of TFIID or TFIIB (review-Roeder, 1996). This induces bending of the promoter DNA, facilitating the recruitment of more TFIIB proteins and is followed by the attraction of hypophosphorylated RNAPII; often pre-assembled with many other GTFs such as TFIIF, TFIIE and TFIIH (Ha *et al.*, 1993; Horikoshi *et al.*, 1992; Nobel lecture-Kornberg, 2007; Nikolov *et al.*, 1996). Thanks to the ATPase and helicase activity of TFIIH, a negative superhelical tension is introduced onto DNA causing its denaturation and thereby formation of an open pre-initiation complex (PIC), where dNTPs start to pair with the template DNA (Holstege *et al.*, 1997). Initially, RNAPII makes several attempts for transcription where large amounts of 3-4nt long RNAs are released (Lescure *et al.*, 1981). After the synthesis of the first 4 nucleotides, a short RNA molecule is stabilised by the B-finger domain of TFIIB and the switch domain of RNAPII (RPB2 subunit) (Majovski *et al.*, 2005; Pal *et al.*, 2005). As new nucleotides are added, growing RNA starts to collide with the TFIIB, inducing mechanical stress inside the complex. Increased stress causes the collapse of the transcriptional bubble after ~10nt, followed by the release of TFIIB and the RNA touching the exit tunnel (Kugel and Goodrich, 2000; Pal and Luse, 2003). Finally, the S5 residue of CTD tail gets phosphorylated by the activity of cyclin dependent kinase 7 (CDK7) which is found in a complex with TFIIH (Komarnitsky *et al.*, 2000; Serizawa *et al.*, 1995; Shiekhataar *et al.*, 1995; Trigon *et al.*, 1998). Furthermore, it induces conformational changes on the PIC provoking effective 'promoter clearance' (review-Hirose and Ohkuma, 2007; Zhang and Corden, 1991).

1.6.3.2 Promoter-proximal pausing and productive elongation

Right after promoter escape, RNA is synthesised more effectively but an early elongation complex (EEC) is still prone to lateral movements and backtracking if the RNA-DNA hybridisation is weak. This usually takes place until the +23 position (Pal and Luse, 2003).

During early elongation, the S5 phosphorylated CTD tail recruits the RNA capping machinery and H3K4 specific methylase SET1, which provides an open chromatin structure [see section 1.3.3.2] (Ho and Shuman, 1999; Ng *et al.*, 2003). Interestingly, somewhere between +20 and +40, RNAPII gets stalled, a phenomenon known as “promoter-proximal pausing”. This stalling effect is linked to the interaction of EEC with two inhibiting factors: DSIF (DRB sensitivity-inducing factor) and NELF (negative elongation factor) (Andrulis *et al.*, 2000; Ping and Rana, 2001; Wu *et al.*, 2005). Both DSIF and NELF were suggested to inhibit transcription by counteracting the nascent transcript at a certain length and thereby determine at which point pausing would take place (Missra and Gilmour, 2010). Importantly, DSIF also interacts with the capping machinery and stabilises 5' RNA capping at this stage of transcription (Wen and Shatkin, 1999).

DNA sequences at the pausing sites have been examined as well and they were found to be CpG rich (Core *et al.*, 2008). Moreover, a ‘CGRWCG’ motif at DPE elements was suggested to serve as a ‘pause button’ (Hendrix *et al.*, 2008; Nechaev *et al.*, 2010). Another study proposes that the position of the +1 nucleosome (first nucleosome after the transcription start site) could determine the localisation of pausing (Mavrich *et al.*, 2008). In line with this, a downstream shift of +1 nucleosome and a nucleosome free region was detected at pausing sites, implying a role for pausing in the maintenance of an open chromatin structure at promoter-proximal regions (Schones *et al.*, 2008).

Release from the pausing occurs after the recruitment of P-TEFb complex (positive transcription elongation factor –b). Details of molecular events triggering P-TEFb recruitment remain unknown but physiological stimuli such as heat shock, interactions with sequence specific factors (e.g. NF- κ B, c-MYC) or association with the short nascent transcripts could be rate limiting factors (Barboric *et al.*, 2001; review-Peterlin and Price, 2006; Rahl *et al.*, 2010). P-TEFb phosphorylates DSIF, NELF and S2 residues of the CTD tail through its CDK9 kinase subunit (Fujinaga *et al.*, 2004; Price, 2000; Yamada *et al.*, 2006). Overall, EEC undergoes conformational changes at this point and matures into a productive elongation complex (EC) via the stimulatory effects of TFIIS on the catalytic activity of RNAPII (Adelman *et al.*, 2005). Soon after, NELF dissociates and this allows further phosphorylation of CTD S2 residues by another kinase (CDK12), whereas DSIF remains associated with the EC and probably exerts stabilising effects on nascent transcripts or recruits further complexes needed for elongation (i.e. PAF1C and TAT-SF1) (Andrulis *et al.*, 2000; Chen *et al.*, 2009; Hartzog *et al.*, 1998; Rahl *et al.*, 2010). Several other proteins such as ELL (eleven-nineteen lysine rich leukemia) and Elongins also participate in the maintenance of stable maturation of the complex (Conaway and Conaway, 1999). Crucially, a mature EC is associated with the

splicing of introns from the nascent transcript by the splicing machinery, which interacts dynamically with the hyperphosphorylated CTD tail (Carty *et al.*, 2000; Morris and Greenleaf, 2000).

Another dimension of productive elongation occurs at the level of chromatin. Elongation is typically characterised with H3K36 methylation, which is catalysed by SET2 homologues recruited by the phosphorylated S2 residues of the CTD tail (Schaff *et al.*, 2003; Xiao *et al.*, 2003). Moreover, H3K79 methylation and H2BK123 ubiquitination were also suggested to have regulatory implications in the maintenance of productive elongation (Krogan *et al.*, 2003; Pavri *et al.*, 2006). H3K79 during transcription is thought to be maintained via the recruitment of DOT1 through an interaction with H2BK123 ubiquitination (Briggs *et al.*, 2002; Ng *et al.*, 2002; review-Nguyen and Zhang, 2011). H2B monoubiquitination by RAD6, which interacts with the hyperphosphorylated RNAPII, was also shown to be a prerequisite for efficient H3K4 methylation (Dover *et al.*, 2002; Ng *et al.*, 2002; Xiao *et al.*, 2005). Interestingly, H2B ubiquitination was also shown to recruit 19S ATPase subunits of the proteasome (in *S. cerevisiae*), which was suggested to aid successive elongation [see section 1.7.2] (Ezhkova and Tansey, 2004; Ferdous *et al.*, 2001). Numerous factors help RNAPII to move through the chromatin. While ATP-dependent chromatin remodelers (mainly SWI/SNF and CHD complexes) maintain a loose chromatin structure, nucleosome disassembly and reassembly takes place via the histone displacement activity of FACT (facilitates chromatin transcription) complex and histone chaperone activity of SPT6 (SH2 domain containing protein) as RNAPII moves along the gene (Belotserkovskaya *et al.*, 2003; Bortvin and Winston, 1996; review-Lusser and Kadonaga, 2003). Due to these factors, efficiently-elongating RNAPII molecules move very fast in a 'Brownian ratchet' mechanism, where new bonds with the growing transcript are made and broken serially (Bar-Nahum *et al.*, 2005).

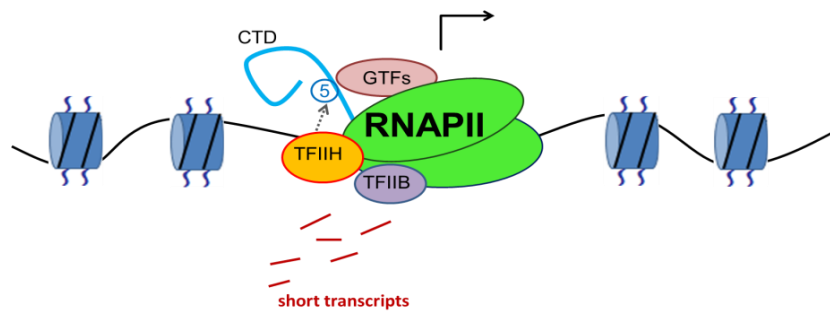
1.6.3.3 Termination

Termination of RNAPII transcription could be maintained by various pathways. These are mainly driven by RNA 3' end processing signals (as a result of DNA sequence) or terminal factors that interact with various components of the transcription machinery [for a recent review, see (Kuehner *et al.*, 2011)]. Two major mechanisms are thought important in higher eukaryotes. In poly(A)-dependent termination, cessation of transcription is coupled with the cleavage of nascent transcript. CPSF (cleavage and polyadenylation specificity factor) recognises a polyadenylation signal (AAUAAA) on RNA and interacts with RNAPII body, whereas its partner CSTF (cleavage stimulatory factor) recognises a downstream GU-rich processing signal and binds to the CTD tail, inducing halting of RNAPII and cleavage of

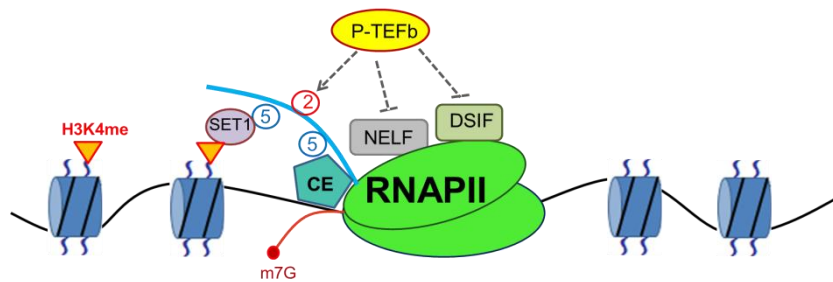
transcript for subsequent polyadenylation (Kazerouninia *et al.*, 2010; Nag *et al.*, 2007; Park *et al.*, 2004). In many cases, RNAPII is somehow released after this point but is stopped again via a 'torpedo' effect created by XRN2 that targets the remaining RNA in a CTD dependent manner via the stabilising action of PCF11 (Kim *et al.*, 2004; West *et al.*, 2004b; Zhang and Gilmour, 2006). Another pathway discovered *in S. cerevisiae* leads to an alternative model where the termination signal is recognised by SEN1 (human homologue: *Senataxin*), which is a helicase that triggers unwinding of the RNA-DNA hybrid (Steinmetz and Brow, 1996). After termination, RNAPII is released and is recycled for further use after phosphorylations of CTD tail are reversed by phosphatases [SCP1 and SSU72 for serine-5, FCP1 for serine-2] (Cho *et al.*, 2001; Krishnamurthy *et al.*, 2004; Yeo *et al.*, 2003). On the other hand, successfully capped, spliced and polyadenylated messenger RNA (mRNA) is exported from the nucleus into the cytoplasm for ribosomal translation (review-Erkman and Kutay, 2004).

Figure 1.17

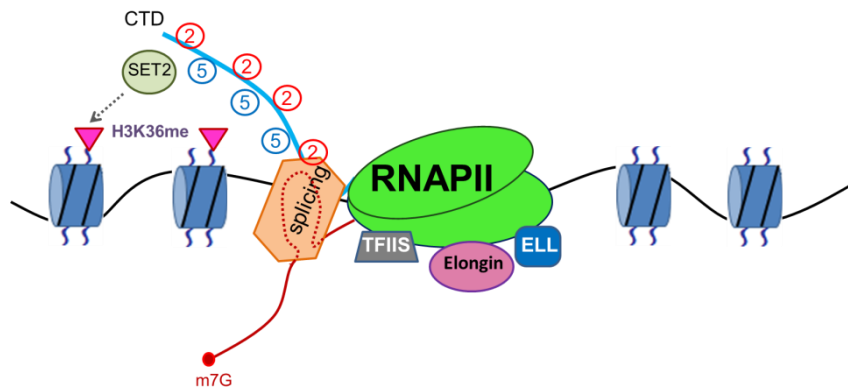
A. initiation



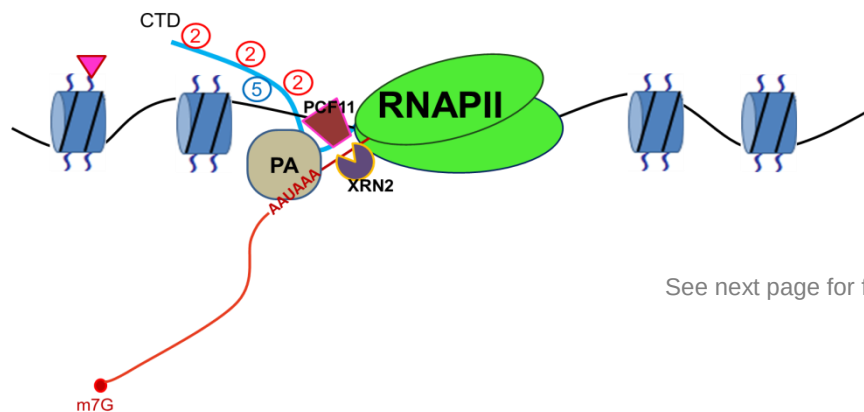
B. promoter-proximal pausing and early elongation



C. productive elongation



D. termination



See next page for figure legend.

Figure 1.17 - Transcription cycle. **[A] Initiation.** General transcription factors (GTFs) bind to the core promoter attracting TFIIB and TFIIF to the site. At this stage, RNAPII makes several attempts for transcription and releases large amounts of 3-4nt long transcripts. TFIIF phosphorylates the CTD tail at serine-5 (S5) residues and triggers promoter clearance. **[B] Promoter-proximal pausing and early elongation.** S5p CTD tail recruits H3K4 specific HKMT SET1. Not long after promoter clearance, RNAPII pauses as a result of NELF and DSIF binding. This coincides with the capping of nascent transcript by the capping enzyme (CE) that interacts with the CTD tail. Release from pausing occurs after P-TEFb phosphorylates serine-2 (S2) residues of CTD as well as DSIF and NELF. **[C] Productive elongation.** RNAPII complex becomes hyperphosphorylated at S2 CTD and matures into a productive elongation complex with the stimulatory effects of TFIIIS, Elongin and ELL. Splicing machinery is attracted by CTD. Productive elongation is associated with the H3K36 methylation mark catalysed by SET2, which is recruited by S2p CTD. Also increased H2BK123 ubiquitination as well as H3K79 methylation was linked to effective elongation (not shown on figure) **[D] Termination.** An AAUAAA signal is recognised by poly-A terminal processing factors and PCF11 followed by the transcript cleavage by exonuclease XRN2.

1.6.4 Functional impacts of RNA polymerase II stalling

Stalling of RNAPII was initially described at the promoter-proximal region of the heat shock gene *HSP70*, where pausing drives loading of RNAPII molecules and prepare the gene for rapid activation upon heat shock stimuli (Brown *et al.*, 1996; Gilmour and Lis, 1986; Sun and Li, 2010). A similar effect was also observed on the human *c-MYC* promoter and the major histocompatibility class II *DRA* gene, where interferon- γ stimulates rapid activation (Eick *et al.*, 1987; Spilianakis *et al.*, 2003). The same phenomenon was also reported to be crucial for the maintenance of the inactive status of the uninduced *P21* gene (Espinosa *et al.*, 2003) and latent-phase HIV-LTR (Kao *et al.*, 1987). Importantly, genome-wide profiling of RNAPII suggested a widespread and conserved role for pausing (Guenther *et al.*, 2007; Kim *et al.*, 2005; Muse *et al.*, 2007; Zeitlinger *et al.*, 2007). Accordingly, a basal level of RNAPII pausing provides a checkpoint for ensuring successful transcription of protein coding genes by precluding positioned nucleosomes at core promoters, as well as giving time for 5' RNA capping and recruitment of splicing factors (review-Li and Gilmour, 2011). Pausing was observed at enhancers as well and this may imply a role in promoter loading via tracking and looping (review-Szutorisz *et al.*, 2005; Xu *et al.*, 2009b). In line with this, paused RNAPII seems to associate with chromatin insulators such as CTCF (review-Li and Gilmour, 2011; Shukla *et al.*, 2011).

In addition to the examples raised above, a distinct form of RNAPII stalling was reported in *Drosophila* and mammalian polycomb (PRC)-repressed genes (e.g. *MSX1*, *MASH1*, *CDX2*, *GATA2* etc.) which are maintained in a silenced state until developmental activation (Breiling *et al.*, 2004; review-Brookes and Pombo, 2009; Stock *et al.*, 2007). In this case, RNAPII is in an unusual 'poised' conformation where the S5p form is detected further downstream of promoters without any sign of the S2p form of RNAPII. In contrast to pausing in stimuli responsive genes, low levels of transcription were also observed in the 'inactive' state (Stock *et al.*, 2007). In terms of physiological relevance, it was suggested that poised RNAPII bookmarks these genes for future activation (review-Levine, 2011). Mechanistically, loss of PRC mediated H2A ubiquitination is thought to release poised RNAPII complexes (Stock *et al.*, 2007). Intriguingly, genes subject to poised RNAPII exhibit both repressive H3K27 and activating H3K4 methylation marks forming a 'bivalent' chromatin (Azura *et al.*, 2006; Bernstein *et al.*, 2006a). Importantly, the balance between CTD specific kinases and phosphatases give rise to distinct profiles for the phosphorylation states of CTD tail, based on various configurations of RNAPII [i.e. 'active', 'paused' or 'poised'] (review-Brookes and Pombo, 2009). Gene profiles of chromatin marks and CTD phosphorylation states exclusive to these specific forms of RNAPII stalling are summarised in figure 1.18.

Studies from bacteria and yeast showed that a productively elongating RNAPII may also stall when obstructed. For example, a head-on collision with the replication fork can stop RNAPII (French, 1992). Also, RNA-DNA hybrids (R-loops) can impede an elongating RNAPII because of the negative supercoiling effect (Grabczyk *et al.*, 2007; Huertas and Aguilera, 2003). Unusual DNA structures caused by repetitive DNA [see section 1.8.3.3] were proposed as a potential blockage as well (review-Everett and Wood, 2004). Consistently, hairpins or loops created by (CNG)_n and (GAA)_n repeats were found to cause RNAPII arrest (Grabczyk *et al.*, 2007; Salinas-Rios *et al.*, 2011). Finally, RNAPII stalls on a DNA lesion as a consequence of direct blockage induced by bulky adducts caused by the damaging factor or an obstructing effect created by pre-bound sensory proteins (review-Hanawalt and Spivak, 2008; Mellon *et al.*, 1987; review-Svejstrup, 2003). Damage-stalled RNAPII signals for components of the DNA repair machineries, an intrinsic mechanism known as transcription coupled repair (TCR) (review-Hanawalt and Spivak, 2008; Mellon *et al.*, 1987). TCR can be coupled primarily with nucleotide excision repair (NER) (Christians and Hanawalt, 1992; Mellon *et al.*, 1987), base excision repair (BER) (Cooper *et al.*, 1997) or in some cases with mismatch repair machineries (Le Page *et al.*, 2000; Leadon and Avrutskaya, 1997). The exact mechanism for how stalled RNAPII triggers TCR is not well-understood yet but studies with human cells suggested that strong binding of ATPase/chromatin remodeler CSB (Cockayne syndrome B) with arrested RNAPII and its interaction with 3' exonuclease XPG (*Xeroderma pigmentosum* G) can initiate NER (review-Hanawalt and Spivak, 2008; Iyer *et al.*, 1996; Levine, 2011; van Gool *et al.*, 1997).

As explained above, stalling of elongating RNAPII can take place in various situations and is generally overcome by elongation factors such as TFIIF, TFIIH, TFIIS, ELL, elongins, CSB and DSIF (review-Sims *et al.*, 2004). In case of TCR, stalled RNAPII may resume elongation via CSB, TFIIH and TFIIS (Mote *et al.*, 1994; Tantin, 1998; Viswanathan and Doetsch, 1998). If read-through does not happen, RNAPII may reverse translocate and backtracked DNA is marked with RPA (replication protein A) for further recognition by repair enzymes (Donahue *et al.*, 1994; review-Hanawalt and Spivak, 2008). Crucially, a persistently stalled RNAPII can trigger apoptosis by P53 induction in human cells (Ljungman and Zhang, 1996; Yamaizumi and Sugano, 1994). Perhaps as a protective feature, durably stalled S2p RNAPII is subject to DEF1-stimulated and UBC5(E2)/ RSP5(E3) [yeast] or NEDD4 [mammals] driven ubiquitination at its large RPB1 subunit, which is subsequently degraded by the 26S proteasome (Anindya *et al.*, 2007; Bregman *et al.*, 1996; Lee *et al.*, 2002; Mitsui and Sharp, 1999; Somesh *et al.*, 2005; Somesh *et al.*, 2007; Woudstra *et al.*, 2002). This degradation is accelerated by hyperphosphorylation of RPB1 when a mammalian cell is exposed to damaging agents such as UV-light (Luo *et al.*, 2001).

Figure 1.18

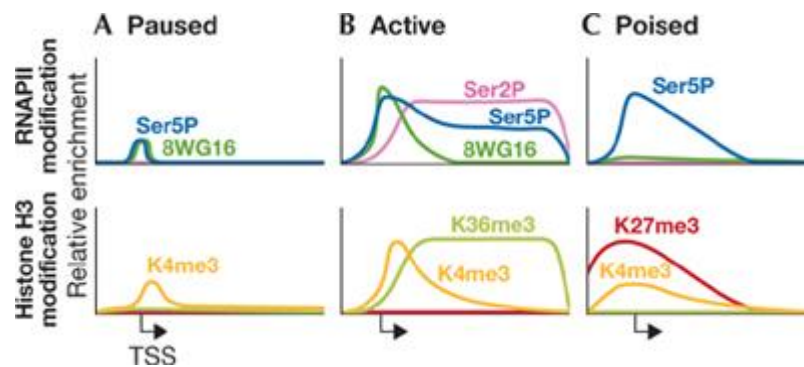


Figure 1.18 - Distinct characteristics of RNAPII and chromatin at 'paused', 'active' and 'poised' genes. **[A] Paused gene.** Stimuli-responsive paused genes such as *HSP70*, all exhibit low levels of S5p and 8WG16 signal (an antibody which recognises unphosphorylated S2 residues) as well as a low level of the H3K4me3 mark in a manner limited to the promoter-proximal region. Consistent with the inactive status, no S2p enrichment is observed. **[B] Active gene.** On the other hand, the peak of S5p is extended throughout the active genes and elevated levels of S2p levels are observed beyond the promoter. 8WG16 signal is lost in the gene body, implying that S2 residues are saturated with phosphorylation. In terms of histone marks, active genes show a higher level and extended distribution of H3K4me3 as well as H3K36me3. **[C] Poised gene.** PRC repressed poised genes typically exhibit a high level of S5p at the promoter and a moderate level of S5p along the gene body. Consistently with their inactive status, no S2p levels are detected. Interestingly, very little or no 8WG16 signal is detected, suggesting that RNAPII could be in an unusual configuration at poised genes. These genes also show an extended but low level of the H3K4me3 mark and a high level of repressive mark H3K27me3, indicating a 'bivalent' state of chromatin. [Adapted from (review-Brookes and Pombo, 2009)]

1.7 The proteasome

1.7.1 Proteasomal degradation of proteins

Protein turnover is vital for the cell and is predominantly maintained by the ubiquitin-proteasome pathway, a highly conserved system from yeast to mammals (review-Bochtler *et al.*, 1999). This involves polyubiquitination of cytosolic or nuclear proteins and their subsequent recognition by the proteasome that catalyses degradation. Importantly, proteasome function is linked to essential mechanisms including protein quality maintenance, DNA repair, transcription, cell cycle, signal transduction and antigen presenting (review-Pickart and Cohen, 2004). Moreover, impairment of ubiquitin-proteasome system was implicated in pathological conditions from cancer to neurodegenerative diseases (review-Mani and Gelmann, 2005; review-Schwartz and Ciechanover, 1999).

Ubiquitin is an evolutionarily conserved 76 amino-acid long peptide, whose attachment to target proteins is a three-step enzymatic process [Figure 1.19A]. Firstly, ubiquitin is activated in an ATP-dependent manner and linked to an active cysteine residue of the ubiquitin-activating enzyme (E1 or UAC) via a thioester bond. This is followed by the transfer of activated ubiquitin to one of the many (over 40) ubiquitin-conjugating enzymes (UBCs, E2) via another thioester bond. Finally, ubiquitin is transferred from E2 to the target protein that is pre-bound with a ubiquitin-ligase (E3) (Ciechanover *et al.*, 1980; Hershko *et al.*, 1983; review-Pickart, 2004). This involves formation of an amide bond with the terminal glycine of ubiquitin and a lysine residue in the target protein. There are more than 500 ubiquitin-ligases in mammals that catalyse either direct (RING finger E3s) or indirect (HECT domain E3s) ubiquitin transfer (review-Kinyamu *et al.*, 2005). E3s recognise their substrate by a consensus N-terminal amino-acid motif whose exposure rate to E3s could be modulated by the protein conformational changes [i.e. post-translational modification] (review-Pickart, 2001). Successive rounds of this cascade results in polyubiquitination via intermolecular ubiquitin glycine-lysine bonds. Notably, whereas polyubiquitination signals for proteasomal degradation, monoubiquitination either acts as a bridge or mark a protein for a certain function, as seen in histone H2A/B (review-Kodadek, 2010). Notably, ubiquitination is dynamic and can be reversed by deubiquitinating enzymes (DUBs). There are more than 90 DUBs identified in mammals (review-Ventii and Wilkinson, 2008).

Although degradation of proteins by proteasomes is largely dependent on a ubiquitination signal, there are also reported cases where ubiquitin is not required for substrate recognition (review-Jariel-Encontre *et al.*, 2008). For example, oxidised or misfolded proteins undergo ubiquitin-independent proteasomal degradation, for which no ATP is needed (Grune *et al.*,

1996; review-Grune *et al.*, 1997; Grune *et al.*, 1995; Shringarpure *et al.*, 2003). Although the exact mechanism is unclear, it is thought that such degradations take place due to the extensive chemical modifications of oxidised proteins or unstable nature of malfoldings, which increase the exposure rates of hydrophobic patches to proteasome subunits (Giulivi *et al.*, 1994; Jariel-Encontre *et al.*, 2008; Pacifici *et al.*, 1993). A classic example is the degradation of ornithine decarboxylase (ODC), which needs the action of protein destabilising antienzyme AZ1 (Bercovich *et al.*, 1989; Glass and Gerner, 1987). Also, some transcription factors including c-Fos, HIF1 α or P53 can be subject to ubiquitin-independent proteasomal degradation either by interacting with destabilising proteins or due to the effect of some post-translational modifications (Asher *et al.*, 2005; Bossis *et al.*, 2003; Kong *et al.*, 2006). The mechanisms behind this phenomenon remain poorly understood. Generally, protein destabilising events are thought to replace ATP dependent activities of proteasome and thereby increase the chance of proteins to be randomly degraded. Also, ubiquitin – independent protein motifs were suggested but these are not identified yet (review-Jariel-Encontre *et al.*, 2008).

Eukaryotic proteasomes are often found as a large (2.5mDa) 26S holoenzyme complex comprising a 19S regulatory particle and a 20S catalytic core particle [Figure 1.19B]. 19S regulatory particle (also known as PA700), which is made up of a ‘base’ and ‘lid’, is responsible for the recognition of ubiquitin signal and directing the substrates into the 20S-core particle. The 19S base contains six ATPases (regulatory particle triple; RPT1-6) that form a pseudosymmetric ring and four regulatory proteins (regulatory particle non-ATPase: RPN -1,-2,-10,-13) (Fu *et al.*, 1999; review-Kim *et al.*, 2011b; Nickell *et al.*, 2009). Mechanistically, ATPases unfold the substrate and allow their translocation into the 20S catalytic chamber by inducing conformational changes for gate opening (Braun *et al.*, 1999; Smith *et al.*, 2007). On the other hand, RPN1/RPN2 provide a docking site for substrate recruitment whereas major ubiquitin receptors RPN10/RPN13 ensure the affinity for the substrate and have structure stabilising properties (Husnjak *et al.*, 2008; Riedinger *et al.*, 2010; Rosenzweig *et al.*, 2008). The 19S lid consists of nine non-enzymatic regulatory proteins (RPN3, RPN5-9, RPN11-12 and RPN15). Most of these have PCI domains, which serve as an interacting scaffold between proteasome, signalosome and eukaryotic translation initiation factor eIF3 (Fu *et al.*, 2001; Isono *et al.*, 2004; review-Kim *et al.*, 2011b; Takeuchi *et al.*, 1999). It has been suggested that lid proteins are also involved in the direct substrate recognition but so far no exact characterisation has been made (review-Kim *et al.*, 2011b; review-Kinyamu *et al.*, 2005). Importantly, RPN11 is a deubiquitinating enzyme that cleaves the ubiquitination signal prior to degradation (Verma *et al.*, 2002; Yao and Cohen, 2002). Moreover, 19S interacts with two other DUBs; UCH37 [through RPN13] and USP14

[through RPN1] (Borodovsky *et al.*, 2001; Stone *et al.*, 2004). In addition to 19S, higher eukaryotes have two 11S regulatory particles. PA28 (in three isoforms α, β, γ) triggers gate opening just like 19S whereas PA200 rather implements a disordered gate structure that only allows small proteins and peptides to undergo degradation (Lehmann *et al.*, 2008; Whitby *et al.*, 2000). 11S activators are known to mediate ubiquitin-independent degradation of proteins; however, how they recognise the substrates remain unclear.

The 20S core particle is a hollow, barrel-like catalytic complex comprised of four stacked rings formed by two outer heptameric α subunits (α_{1-7}) and two inner heptameric β subunits (β_{1-7}) in a $\alpha_{1-7}-\beta_{1-7}-\beta_{1-7}-\alpha_{1-7}$ manner (Groll *et al.*, 1997; Kwon *et al.*, 2004; Lowe *et al.*, 1995). Once placed inside the catalytic chamber, substrate proteins are cleaved into small peptides via the proteolytic activities of β_1 (caspase-like), β_2 (trypsin-like) and β_5 (chymotrypsin-like) subunits (Arendt and Hochstrasser, 1997; Heinemeyer *et al.*, 1997). Although independent 20S core particles can be found within the cell, their conformation does not allow substrates to enter the catalytic chamber (Groll *et al.*, 2000). Therefore, unregulated proteolysis is strongly prohibited and degradation is dependent on regulatory particles. All 19S and 11S regulatory complexes can be freely found in the cell but the mechanism as to how and when exactly these associate with the 20S particle remains largely to be investigated (review- Stadtmueller and Hill, 2011). Several studies point out a role for proteasome post-translational modifications (i.e. phosphorylation and glycolysation), which not only affect assembly but also substrate binding and ATPase activities (Davy *et al.*, 2001; Kikuchi *et al.*, 2010; Mason *et al.*, 1996; Satoh *et al.*, 2001). Also additional proteins such as PAAF1 (proteasome ATP-ase associated factor 1) could play a regulatory role for the assembly of 26S proteasomes (Park *et al.*, 2005).

A.

Ub

E1

ATP

AMP + P_{pi}

E2

E3

substrate

proteasome

19S

20S

19S

oligopeptides

UAC

UBC2/RAD6/HR6A/B
UBCH5
UBCH7
UBC9

E6AP,MDM2,RSP5,
CHIP,TBL1,TBLR1,
SKP1ab,BRCA1,
TAFII250,
RING1A/B

B.

19S RP

Lid: Sem1, Rpn 3,
Rpn 5-9, 11, 12

Rpn10

Rpn1, 2, 13

Rpt-ring

20S CP

Heteromeric rings

5 1
4 2
3 6

Rpt-ring

β_7 β_8 β_9
 β_1 β_2 β_3 β_4
 β_5 β_6

β -ring

α_7 α_6 α_5 α_4
 α_1 α_2 α_3

α -ring

19S RP

Rpn10

Rpn1, 2, 13

Lid: Sem1, Rpn 3,
Rpn 5-9, 11, 12

1.7.2 Proteasome inhibitors

The involvement of proteasome in many subcellular pathways led to the development of its chemical inhibitors. More than 50 inhibitory molecules with a dozen different mechanisms of action have been identified so far (review-de Bettignies and Coux, 2010). In this Ph.D. study, two well-established inhibitors, MG132 and PS341 were used. Both of these molecules form reversible complexes with the catalytic threonine hydroxyl groups of 20S core particle mimicking the transition state during peptide-bond cleavage (Groll *et al.*, 2006; Zhang *et al.*, 2009). This means that the effects are temporary based on the presence of inhibitor. Another crucial feature is that both of these drugs have cell cycle inhibitory affects with great potential as chemotherapeutic agents (Adams *et al.*, 1999; Chauhan *et al.*, 2005; Drexler, 1997; Landowski *et al.*, 2005; Yan *et al.*, 2007).

MG132 (Z-Leu-Leu-Leu-al) is a C-terminal peptide aldehyde and a competitive substrate analogue, that inhibits the activity of all β 1, β 2 and β 5 catalytic subunits [Figure 1.20A] (Berkers *et al.*, 2005; Tsubuki *et al.*, 1993). It has a strong inhibitory potential and acts in a highly selective manner unlike many other aldehyde inhibitors, and therefore does not interfere with other cellular processes (Bogyo *et al.*, 1997). The three leucine molecules plus a blocking group on the N-terminus promotes high cell permeability for MG132 (Rock *et al.*, 1994; Tsubuki *et al.*, 1993). The strong potential, high selectivity and cheap price makes MG132 a valuable tool for experimental approaches, and indeed it was used in numerous high impact studies [e.g. (Palombella *et al.*, 1994; Rock *et al.*, 1994)]. IC₅₀ for MG132 is 0.024 μ M for the chymotrypsin-like (β 5) activity, 9.215 μ M for trypsin-like (β 2) activity and 2.288 μ M for the caspase-like (β 1) activity *in vitro* (Braun *et al.*, 2005). MG132 has typically been used at a concentration of 10 μ M in cell culture studies (Maddika *et al.*, 2011; Malureanu *et al.*, 2009; Zhu *et al.*, 2008). Although there are some examples of MG132 *in vivo* administration [e.g. (Inoue *et al.*, 2009; Jamart *et al.*, 2011)], no systematic toxicology and pharmacokinetics studies have been performed for this drug so far.

PS341 (Pyz-Phe-boroLeu, Bortezomib, Velcade®) is a dipeptide molecule derived from competitive aldehyde inhibitors by replacing the carboxylic acid of leucine residue with boronic acid, which highly increases the specificity and efficiency of proteasome inhibition [Figure 1.20B] (Adams *et al.*, 1998; Groll *et al.*, 2006). It has a high cell permeability like classical aldehyde inhibitors but a prolonged chemical stability (review-Richardson *et al.*, 2006). PS341 targets the β 1 activity to a low extent and β 5 activity to a higher extent (Berkers *et al.*, 2005; Groll *et al.*, 2006). High selectivity, cell cycle inhibitory effects and relatively low toxic effects of PS341 in animal studies, made it reach clinical use as the first proteasome inhibitor (review-Richardson *et al.*, 2006). Indeed, inhibition of proteasome by

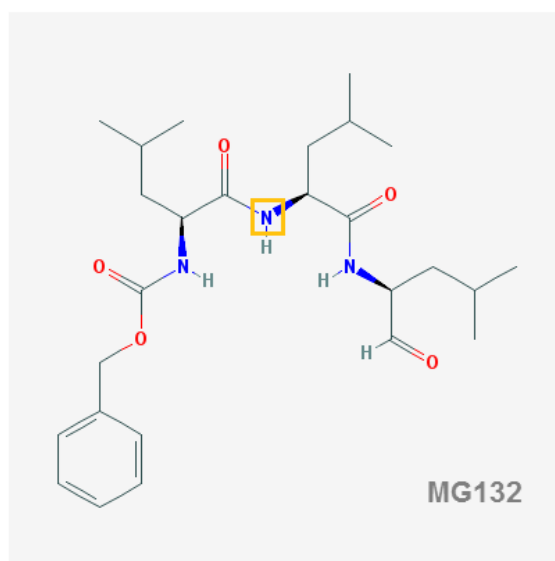
PS341 made a great impact on the treatment of multiple myeloma (Hideshima *et al.*, 2001; review-Shah and Orlowski, 2009). In this case, PS341 suppresses NF- κ B, which is involved in key events during the development of multiple myeloma, by stabilising the inhibitory molecule I κ B (Annunziata *et al.*, 2007; Hideshima *et al.*, 2001). Other effects of PS341 include stabilising pro-apoptotic P53 and BAX proteins (Lee *et al.*, 2003; Mitsiades *et al.*, 2002), dissipating the mitochondrial membrane potential as well as inducing release of mitochondrial cytochrome c (Ling *et al.*, 2003), activating c-Jun-N terminal kinase (Lee *et al.*, 2003; Mitsiades *et al.*, 2002), stimulating endoplasmic reticulum stress (Fribley *et al.*, 2004) and immunoglobulin production (Meister *et al.*, 2007). Moreover it has depleting effects on murine lymphocyte development (Maseda *et al.*, 2008).

Biochemical kinetics studies on PS341 reported an *in vitro* IC₅₀ for the caspase-like (β 1) and chymotrypsin-like (β 5) are 0.053 μ M and 0.0079 μ M, respectively (Chauhan *et al.*, 2005). *In vitro* studies reported significant effects of PS341 mediated proteasome inhibition at a wide dose range, 0.1nM to 50 μ M (Fournier *et al.*, 2010; Guan *et al.*, 2009; Hideshima *et al.*, 2001; Larsson *et al.*, 2010; review-Shah and Orlowski, 2009; Shanker *et al.*, 2008). Pharmacokinetics studies concluded a $\sim$ 10 hours half-life and 1.3 mg/m² ($\sim$ 0.5 mg/kg in mouse and $\sim$ 0.03 mg/kg in human) plateau dose with maximum effect within $\sim$ 1 hour (review-Leveque *et al.*, 2007; Orlowski *et al.*, 2002; Papandreou *et al.*, 2004). Animal studies reported a maximum effective and safe dose of $\sim$ 0.75 mg/kg in mice, with LD₅₀ values considered between 1.5-2.0 mg/kg (Cusack *et al.*, 2001; Hamilton *et al.*, 2005; Ishii *et al.*, 2006; Maseda *et al.*, 2008; Neubert *et al.*, 2008). PS341 was reported to cross blood-brain barrier only at a low level (Hemeryck *et al.*, 2007).

There are moderate to severe PS341 side effects in humans which include fatigue, weakness, gastrointestinal disturbances, thrombocytopenia and peripheral neuropathy (Richardson *et al.*, 2003). It is for sure that PS341 opened a new avenue in medicine and hence many more proteasome inhibitors (e.g. carfilzomib, epoxomicin) possibly with higher potential and less side effects have been developed and are waiting for clinical approval (review-Kling, 2010; review-Ruschak *et al.*, 2011).

Figure 1.20

A.



B.

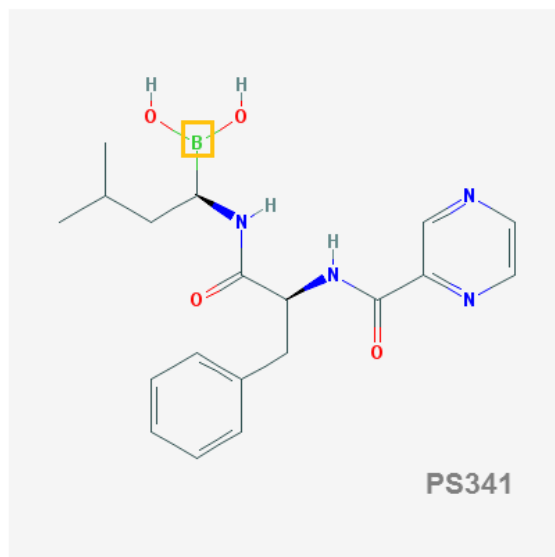


Figure 1.20 - Proteasome inhibitors MG132 and PS341. Both of these molecules are competitive substrate analogues that attack the hydroxyl groups of N-terminal threonines at the proteasome catalytic pockets. **[A] MG132.** This is a classical peptide aldehyde inhibitor. It has a potential inhibiting all catalytic subunits of the proteasome reversibly via forming hydrogen bonds between the N2 (yellow box) molecules and oxygen atoms from threonine-21 residues. **[B] PS341.** This is a boronic dipeptide derived from peptide aldehyde inhibitors. Boron atom (yellow box) replaced with the carboxyl group of leucine strongly attacks threonine-1 residues of $\beta 1$ and $\beta 5$ subunits forming a covalent bond with the nucleophilic oxygen lone pair, releasing H_2O . [Chemical structures obtained from PubChemCompound, <http://www.ncbi.nlm.nih.gov/pccompound>]

1.8 Repetitive DNA and gene regulation

1.8.1 General information

The majority of the higher eukaryotic genome is known to be occupied by non-coding repetitive DNA (review-Charlesworth *et al.*, 1994). More specifically, only ~25% of the human genome comprises protein coding genes, whereas ~55% is repetitive DNA and ~20% is intergenic sequences (Lander *et al.*, 2001). Although repeats were often interpreted as the evolutionary 'junk' material, a lot of evidence now suggests their active contribution to genome function in various aspects including maintenance of genome architecture, serving as a tool for evolution and regulating gene expression. Importantly, unusual placement or spreading of repetitive DNA could disrupt normal gene expression patterns or introduce mutations, and thereby result in a number of pathological conditions. Interestingly, heterochromatin was proposed to have evolved as a protective response to prevent disruptive effects of repeat elements and their contribution to transcriptional noise (review-Bashkirov, 2002; Yoder *et al.*, 1997)

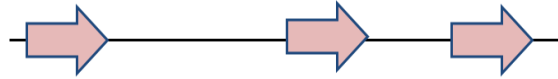
Repeat elements are classified in two main groups. Transfer RNA (tRNA) genes, gene paralogues and all transposons are known as "interspersed repeats" because of their randomly scattered pattern throughout the genome. Interspersed repeats constitute the vast majority of repetitive DNA. 'Tandem repeats', on the other hand, are less pronounced in the genome and they are lined up in arrays where repeat units are linked to each other in a head-to-tail organisation. Tandem repeats comprise gene tandems, ribosomal DNA (rDNA), telomeric repeats and satellite DNA (review-Richard *et al.*, 2008). Repeat elements show distinct chromosomal distribution patterns. Briefly, classical satellites are found in centromeric and pericentromeric regions, whereas mini- and micro-satellites as well as transposons are primarily enriched on chromosomal arms. Moreover, chromosome ends are known to be capped by telomeric repeats. A summary for the overall structure and chromosomal locations of repeat elements is given in figure 1.21.

This thesis partly focuses on transposons and satellite repeats. Therefore their basic structure and contribution to mammalian gene regulation will be discussed in the following sections.

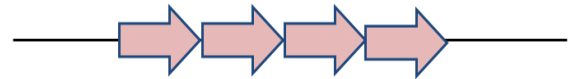
Figure 1.21

A.

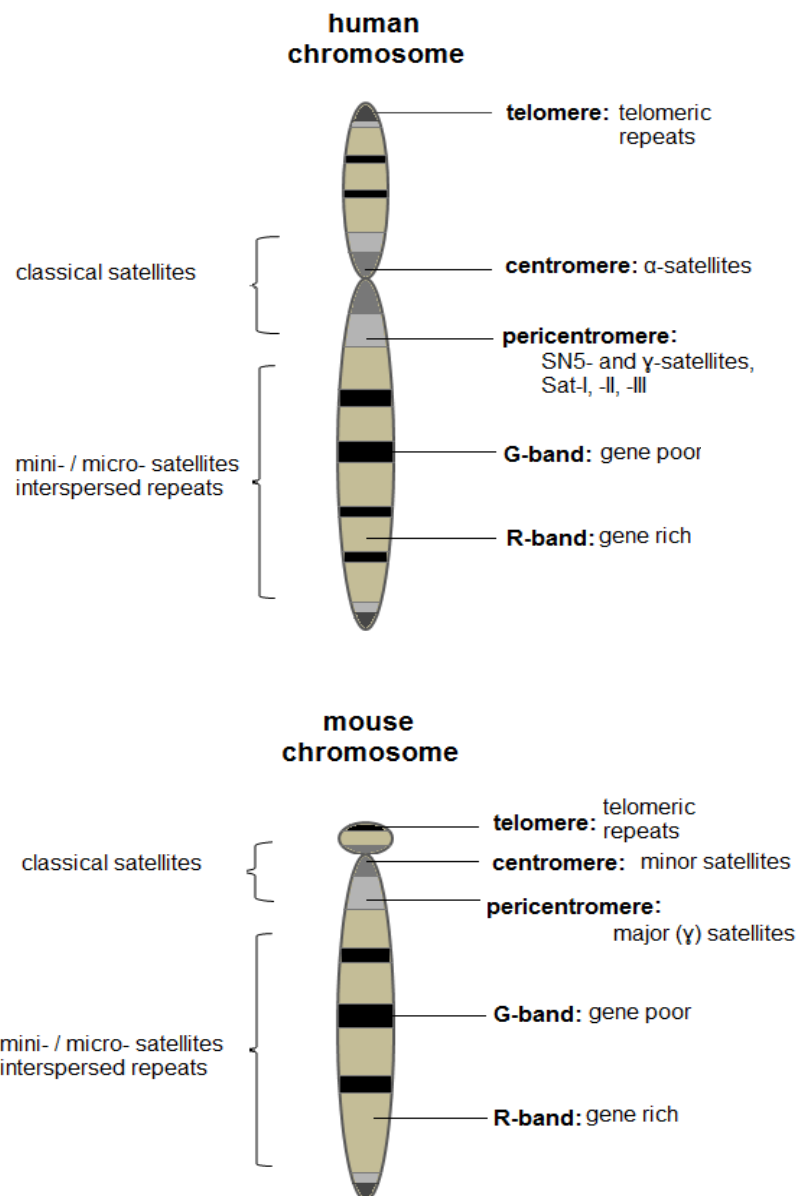
Interspersed repeat: tRNA genes, gene paralogues,
transposons (LINE, SINE ...etc.)



Tandem repeat: gene tandems, rDNA genes,
telomeric DNA, satellite DNA



B.



See next page for figure legend.

Figure 1.21 - A summary for the overall organisation and chromosomal locations of repeat elements. [A] **Organisation of repeat elements.** Interspersed repeats are randomly scattered within the genome and comprise tRNA genes, gene paralogues and transposons. Tandem repeats constitute arrays in a head-to-tail organisation and include gene tandems, rDNA genes, telomeric and satellite DNA. [B] **A simplified diagram for primary locations of repeat elements on human and mouse chromosomes.** Classical satellites are found in centromeric and pericentromeric regions, mini- / micro- satellites primarily reside on chromosomal arms, finally telomeric repeats constitute chromosome ends. Centromeres of human chromosomes are known to be enriched by α -satellites, whose mouse counterparts are minor satellites. Pericentromeres, on the other hand, comprise various satellite elements including SN5-, γ -satellites and Sat-I, -II, -III in human and major (γ) satellites in mouse. G-bands are positively stained bands after Giemsa staining. G-bands are typically AT rich and gene poor. R-bands, on the other hand, are unstained or lightly stained regions after Giemsa procedure and comprise gene rich sequences with a high GC content. [Based on (review-Charlesworth *et al.*, 1994; review-Lander *et al.*, 2001; Martens *et al.*, 2005)]

1.8.2 Transposons

1.8.2.1 Structure and maintenance

Transposable elements (transposons) are widespread DNA repeats which can move themselves or 'paste' their copies into new genomic positions (McClintock, 1950). This 'jumping' effect takes place via different mechanisms depending on the type of transposon. Retrotransposons use an RNA intermediate during this 'copy and paste' process whereas DNA transposons are able to 'cut and paste' themselves without the need to be transcribed.

Long interspersed elements (LINEs)

Comprising the highest fraction of genomic DNA, long interspersed elements (LINEs) are a common form of retrotransposons. LINEs are 5-10kb long and they are transcribed by RNAPII. The 5' UTR of LINEs is recognised as a promoter and their 3'UTR contains a polyadenylation (poly[A]) signal [Figure 1.22A] (review-Belancio *et al.*, 2008). LINEs typically consist of two open reading frames (ORFs) which encode an endonuclease and a reverse transcriptase. Briefly, the endonuclease nicks the target AT-rich DNA sequence and reverse transcriptase synthesises cDNA from the LINE mRNA, whose poly(A) tail is primed with the T-rich sequence at the target DNA nick (Jurka, 1997; review-Jurka *et al.*, 2007; Luan *et al.*, 1993). Next, second strand synthesis and ligation are performed resulting in the integration of the copied LINE element into the target DNA sequence. How specifically these later steps take place remains elusive, but it has been proposed that double-strand break repair mechanisms could play a significant role (review-Belancio *et al.*, 2008). LINE elements have several subclasses such as L1, L2 or L3 based on the homology of the reverse transcriptase they encode. Among them, L1 is the predominant LINE element within the mammalian genome (review-Richard *et al.*, 2008).

Short interspersed repeats (SINEs)

Other important members of the 'copy and paste' retrotransposons are short interspersed repeats (SINEs). These elements are shorter than 500bp and known as the most repeated elements within the mammalian genome [Figure 1.22B] (review-Richard *et al.*, 2008). SINEs are short pseudogenes being transcribed by RNAPIII. These elements do not encode any protein and therefore utilise the LINE machinery for integrating themselves into new positions (Dewannieux *et al.*, 2003). SINEs are mostly represented by Alu and MIR (mammalian interspersed repetitive) elements in primates and by B1, B2, ID as well as MIR elements in rodents. Alu repeats, whose rodent counterpart is the B1 repeat, are derived from the 7SL component of the signal recognition particle whereas other SINEs are derived from tRNA genes (Daniels and Deininger, 1985; review-Kramerov and Vassetzky, 2005; Ullu

and Tschudi, 1984). Alu and B1 are known as the most active and abundant forms of SINEs (review-Zamudio and Bourc'his, 2010).

LTR retroelements

LTR retroelements are retrotransposons containing sequences from endogenous retroviruses. These elements typically contain long terminal repeats (LTRs), which serve as promoter and termination signals for RNAPII [Figure 1.22C]. LTR retrotransposons contain several ORFs which encode retroviral proteins such as group specific antigen (gag), protease (PR), integrase (IN) and reverse-transcriptase-RNase-H (RT-RH) (review-Havecker *et al.*, 2004). They usually lack the retroviral envelope protein, which enables a virus to infect other cells. Unlike non-LTR retrotransposons, the priming of reverse transcription usually involves annealing with a tRNA. Integration to DNA is then performed by the integrase (IN) and takes place in a similar way to non-LTR retrotransposons (review-Deininger and Batzer, 2002; McCarthy and McDonald, 2004). There are several sub-classes identified within this family based on the homology of proteins they encode. These include ERV (endogenous retroviral) classes I and II; and MaLRs [mammalian apparent LTR-retroelements] (review-Wicker *et al.*, 2007). The latter one lacks homology with retroviral reverse transcriptases (Smit, 1993).

DNA transposons

The second major class of transposons are DNA transposons which are able to move themselves into new genomic locations with a 'cut and paste' mechanism, which does not involve a RNA synthesis step. These elements are recognised by the self-encoded transposase enzyme at the inverted repeats flanking on either side of the repeat element [Figure 1.22D] (review-Feschotte and Pritham, 2007). Transposases can use different mechanisms in the way they perform the movement of DNA transposons. Generally speaking, these proteins are able to synapse with each other, cleave the donor strand and ligate it to the target strand (Reznikoff, 2003). DNA transposons are classified into 10 different families based on the homology of the transposases they encode. Among them, MER1 (hAT/Charlie) and MER2 (Mariner /Tc1) represent the most common families (review-Wicker *et al.*, 2007).

Figure 1.22

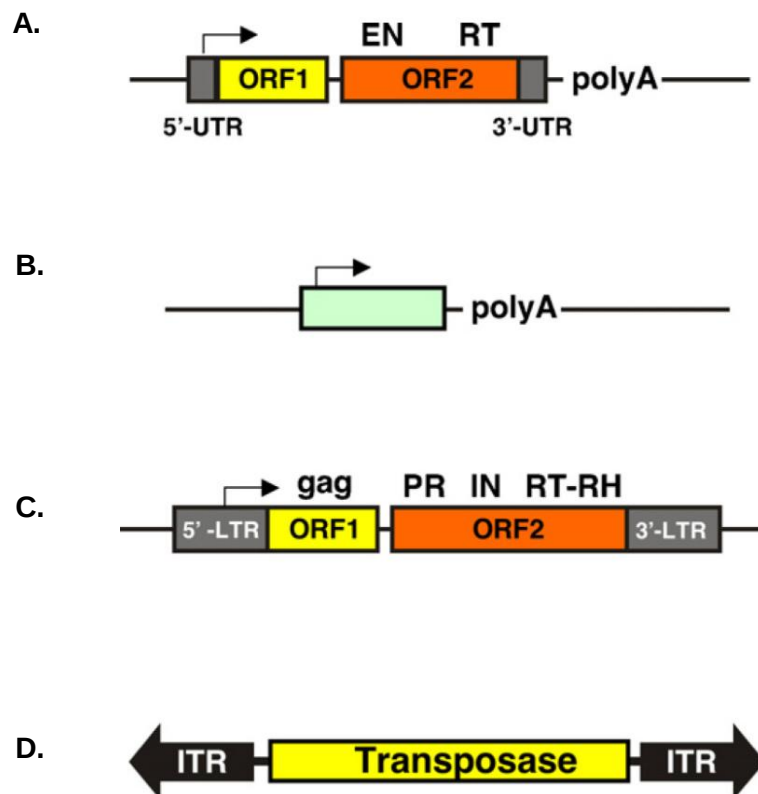


Figure 1.22 - Structural organisation of common transposons. [A] **LINE element.** LINEs consist of a 5' UTR which serves as a promoter, and two ORFs, which encode an endonuclease (EN) and a reverse transcriptase (RT) in order to generate copies of themselves and paste to target DNA sequences. [B] **SINE element.** SINEs are small RNA derived pseudogenes and use LINE machinery for their transposition. [C] **LTR retrotransposon.** These elements generally encode many proteins needed for a retroviral life cycle including gag, protease (PR), integrase (IN), reverse transcriptase and RNaseH (RT-RH). They are able to 'copy' and 'paste' themselves into new genomic regions in one cell but unable to infect other cells since they usually do not encode an envelope protein. [D] **DNA transposon.** Inverted terminal repeats (ITR) elements reside on both sides of a DNA transposon and are recognised by the self-encoded transposase enzyme, which catalyses their 'cut' and 'paste' transposition into target sequences. [Adapted from (editorial-Ivics and Izsvak, 2010)]

1.8.2.2 Transposons and chromatin

Transposons are often associated with defective mutations when integrated into promoters or coding regions of genes. Several diseases caused by such insertions have been identified. To name a few, L1 insertions into crucial genes were shown to trigger β -thalassemia and colon cancer as well as some X-linked diseases including haemophilia and Duchenne muscular dystrophy. Moreover, insertions of Alu elements are known to cause cystic fibrosis, breast cancer, leukemia, neurofibromatosis and also haemophilia (review-Belancio *et al.*, 2008). In addition to this, globally high expression levels of transposons are interpreted as hallmarks of aging and cancer (Barbot *et al.*, 2002; review-Schulz *et al.*, 2006).

In order to cope with the dangerous effects of transposons, cells are thought to have evolved several mechanisms (review-Zamudio and Bourc'his, 2010). In fact, heterochromatin mediated silencing is thought to be the major host response (review-Grewal and Jia, 2007). In fission yeast, silencing of repeat elements were shown to be achieved by RNA interference mediated heterochromatinisation (Volpe *et al.*, 2002). The direct involvement of RNA interference pathway for the silencing of mammalian transposable elements has also been addressed in several studies but there is still no substantial evidence suggesting conservation of this mechanism in mammals [see section 1.4.2] (review-Beisel and Paro, 2011; review-Bernstein *et al.*, 2007; review-Huda and Jordan, 2009). On the other hand, DNA methylation was shown to be the primary driving factor for the silencing of transposons in mammals and their hypomethylation has been linked to several cancers (review-O'Donnell and Burns, 2010; Rollins *et al.*, 2006; review-Yoder *et al.*, 1997). Transposable elements are also thought to affect the chromatin environment by recruiting specific histone modifications. One study, which is performed with human cells, revealed that Alu elements are enriched in H3K9me2 (Kondo and Issa, 2003). Also, LTR transposons in mouse were shown to carry H3K9me3 and H4K20me3 heterochromatin marks (Mikkelsen *et al.*, 2007). This reinforces the notion that mechanisms have evolved to reduce the deleterious effects of transposons. On the other hand, another study concluded that overall modification patterns for transposable elements vary greatly between different cell types in mouse (Martens *et al.*, 2005). Thus, it is possible to speculate that transposable elements do not demarcate genomic regions for one specific effect but instead may have regulatory roles in the regulation of nearby genes, depending on the cellular context or stimuli.

1.8.3 Satellite repeats

1.8.3.1 Main features

Satellite elements are characterised by distinct banding patterns localised in density gradient assays. Due to their unusual nucleobase frequencies (A+T/G+C content), they are sedimented above or below the bulk genome and therefore named as “satellite” DNA (review-Richard *et al.*, 2008; Walker, 1971). These tandem repeat elements can occur in array blocks in the genome or sometimes can be interrupted with other non-coding sequences. It is noteworthy that the satellite repeat sequence and number of repeated patterns exhibit polymorphisms within a given species making these elements a valuable tool for DNA fingerprinting (review-Gemayel *et al.*, 2010).

Satellite repeats can be classified in three categories based on the length of the repeated unit (Charlesworth *et al.*, 1994; review-Richard *et al.*, 2008). These are classical satellites, minisatellites and microsatellites.

1.8.3.2 Classical satellites and heterochromatin

Classical satellites are primarily located within the centromeres and their proximal regions known as ‘pericentromeres’. These elements are highly repetitive and comprise repeat units that are typically 100-300bp in length. Non-coding RNAs could be generated as a result of their transcription by RNAPII in mammals. These transcripts were reported to be recognised by heterochromatin machineries, which propagate their silencing making them the main component of constitutive heterochromatin (review-Eymery *et al.*, 2009). The most striking example is perhaps the recruitment of RNAi mediated heterochromatin machinery in yeast [see section 1.4.2]. However, conservation of this mechanism in mammals is still under debate as mentioned before. Still, it is intriguing to note that accumulation of satellite transcripts was reported in Dicer-deficient mouse embryonic stem (ES) cells (Kanellopoulou *et al.*, 2005). Although RNAi pathways could be a potential explanation for a positive feedback loop for the enrichment of heterochromatin marks, *de novo* targeting of satellites is more likely to be a result of satellite transcript recognition by HP1- α (Maison *et al.*, 2011). Generally speaking, most types of cells exhibit hypoacetylation and enriched H3K9, H3K27 and H4K20 methylation levels, as well as binding of HP1 proteins at classical satellite repeats (review-Guenatri *et al.*, 2004; Jenuwein and Allis, 2001; Maison *et al.*, 2002; Martens *et al.*, 2005; Peters *et al.*, 2001). DNA methylation could be another potential trigger of heterochromatin at these repeats as inhibitors of DNA methylases increased their transcription in mouse erythroleukemic cultured cells (Bouzinba-Segard *et al.*, 2006). Notably, on-going satellite transcription had been detected in mouse ES cells despite

displaying high levels of heterochromatin marks (Martens *et al.*, 2005). Moreover, numerous research studies addressed fluctuating levels of mammalian satellite transcription at different points of the cell cycle and during cellular differentiation processes (e.g. myocyte development) or as a response to cellular stress (e.g. heat shock, DNA damage, etc.) (review-Eymery *et al.*, 2009). Importantly, over-expression of classical satellites has been linked with pancreatic cancer and other epithelial cancers both in mouse and human cells (Ting *et al.*, 2011).

It is thought that heterochromatinisation of classical satellites is a crucial phenomenon for maintaining the nuclear architecture of a given cell (review-Fisher and Merckenschlager, 2002). Knockout studies in yeast and mouse revealed that the maintenance of this heterochromatin is essential for the spatial organization of centromeres and hence efficient segregation of sister chromatids during cell division (Guenatri *et al.*, 2004; Maison *et al.*, 2002; Peters *et al.*, 2008). In addition, a recent study reported ectopic over-expression of classical satellites in cultured mouse and human epithelial cells caused genomic instability characterised by growth arrest, impaired homologous recombination and spontaneous DNA breaks (Zhu *et al.*, 2011). The same study also uncovers a satellite-repressive function of BRCA1, a RING-finger protein involved in DNA repair and known to cause breast cancer when mutated (review-Venkitaraman, 2002, 2009). Accordingly, BRCA1 maintains heterochromatinisation of classical satellites through ubiquitination of H2A via a yet-unidentified mechanism.

Although there are many different classical satellite sequences reported, some of them received particular attention throughout the literature (Jurka, 2000; Jurka *et al.*, 2005). For example, α -satellites in human and minor satellites in mouse constitute the main component of functional centromeres on all chromosomes by depositing the histone variant CENP-A and providing binding sites for the centromere protein CENP-B (Earnshaw and Rothfield, 1985; Masumoto *et al.*, 1989; Schueler and Sullivan, 2006; Willard, 1985). These interactions were reported to initiate rotational positioning of nucleosomes and help the formation of centromere-specific chromatin structure needed for kinetochore formation (Ando *et al.*, 2002; Schueler and Sullivan, 2006; Tanaka *et al.*, 2005). Other important classical satellites in human include β -satellite repeats, which are located on the short arms of acrocentric chromosomes; and pericentromeric SN5- and γ -satellites (Johnson *et al.*, 1992; Lee *et al.*, 2000; Lin *et al.*, 1993; Waye and Willard, 1989). While there is not much known about the functional roles of β - and SN5-satellites, γ -satellites were shown to maintain an intermediary heterochromatin structure that allows some transcriptional processivity. This seems to be achieved by the recruitment of the insulator protein CTCF, making human γ -

satellite repeats a boundary element against further spreading of heterochromatin from pericentromeres into the coding regions (Kim *et al.*, 2009). In mouse, pericentromeric regions are known to be enriched with major satellite repeats (also known as mouse γ -satellite) that attract HP1- α , which is thought to initiate the heterochromatic 'chromocentre' (Guenatri *et al.*, 2004; Joseph *et al.*, 1989). Notably, loading of cohesin molecules also occurs at pericentromeres prior to cell division and this was linked to the SUV39H-HP1 mediated pericentromere silencing in mouse (Koch *et al.*, 2008; review-Peters *et al.*, 2008).

1.8.3.3 Mini-/micro- satellites and their contribution to disease

Other members of tandem satellite family are minisatellites, whose repeat unit could be 7-100bp long, and microsatellites, which have 1-6bp long repeat patterns (review-Charlesworth *et al.*, 1994). Originally, these satellite elements are thought to be evolved by point mutations, small insertions or deletions (Boan *et al.*, 2004; Messier *et al.*, 1996). Also, transposable elements (particularly Alu) were suggested as a primary source for these repeats since they are likely to leave traces of repetitive sequences after transposition (Arcot *et al.*, 1995; Jurka and Gentles, 2006). In terms of genomic distribution, mini- and microsatellites are both moderately repeated within the genome and frequently found in euchromatic regions unlike classical satellites. Whereas minisatellites lie predominantly within untranscribed non-coding regions, microsatellites generally reside within intragenic sequences (review-Richard *et al.*, 2008).

Mini- and microsatellites are known to have a high tendency for repeat expansion mutations and display hypervariable numbers among individuals. Therefore, they are also referred as VNTR (variable number of tandem repeat) elements (review-Gemayel *et al.*, 2010). In order to understand the molecular mechanisms behind the expansions, one should consider unusual DNA conformations arising from these elements (review-Mirkin, 2007). Common examples of these structures are illustrated in figure 1.23A. For instance, (CNG)_n repeats form hairpin-like structures involving both Watson-Crick and mismatched base pairs (Gacy *et al.*, 1995). Notably, RNA-DNA hybrids (known as R-loops) were also reported recently for (CNG)_n repeats (Lin *et al.*, 2010). Moreover, tetrahelical structures were found to be introduced by G quartets induced by (CGG)_n, (CCG)_n and (CGCG₄CG₄)_n repeats (Fry and Loeb, 1994; Usdin and Woodford, 1995). (GAA)_n repeats, on the other hand, are known to cause triplex DNA structures (known as "H-DNA" and "sticky DNA") under the influence of negative supercoiling (Gacy *et al.*, 1998; Sakamoto *et al.*, 1999). Lastly, (ATTCT)_n•(AGATT)_n repeats can also introduce negative stress and cause unwinding of DNA (Potaman *et al.*, 2003). Loss of stabilising interruptions may favour these unstable structures, which are thought to cause strand-slippage during replication and trigger the initial expansion events.

Strand slippage is caused by the mispairing of the template and nascent DNA strands. If the hairpin 'loops out' from the template strand, a contraction takes place after replication. On the other hand, if the loop occurs on the nascent strand, an expansion of the repeat element arises [Figure 1.23B] (review-Mirkin, 2007). Epigenetic factors and mutations which may change the mode of replication fork progress were also suggested as primary driving factors for these early expansions (review-Cleary and Pearson, 2005; review-Mirkin and Smirnova, 2002). Once they reach a threshold level, expansions are even more likely to occur. These late expansion events may be triggered by DNA replication, repair or recombination mechanisms. During replication, unusual DNA conformations can induce strand-slippage as well as replication fork stalling, which could be followed by replication reversal and restart (Dere *et al.*, 2004; Fouche *et al.*, 2006; Sakamoto *et al.*, 2001a). In terms of DNA repair, mismatch repair proteins MSH2 and MSH3 are known to bind to hairpin structures which often inhabit numerous mismatches (Kovtun and McMurray, 2001; Owen *et al.*, 2005). However; in this case, components of the repair machinery are known to stabilise hairpins instead of repairing mismatches. This is thought to favour hairpins and strengthens the possibility of repeat expansion by strand-slippage (review-Mirkin, 2007). Finally, increased recombination rates and unequal cross-over between homologous chromosomes caused by microsatellites were reported as another source for expansions (Jakupciak and Wells, 1999; Napierala *et al.*, 2004; Warren, 1997).

Importantly, mini- and microsatellites were associated with various repeat-induced neurological diseases when the repeat number exceeds a certain threshold level. This may take place in germline development and can be transferred to the progeny, or could be triggered during embryonic development. The fact that microsatellites are mostly located within transcribed genes makes them more likely to dysregulate genes. Neurological pathologies and responsible repeat expansions are summarised in figure 1.23C. The disrupting effects of these repeats could be exerted by various mechanisms, depending on the specific nature of the expanded repeat element and its position along the affected gene.

To name a few, a $(C_4GC_4GCG)_n$ minisatellite expansion ($n > 30$ repeats) at the promoter of the gene encoding neutral cysteine protease CSTB is thought to cause progressive myoclonic epilepsy 1 (EPM1) by disrupting the spacing of promoter elements via tetrahelical structures and thereby reducing levels of CSTB expression (Laloti *et al.*, 1997; Pennacchio *et al.*, 1996; Saha and Usdin, 2001). Also, expanded $(CGG)_n$ repeats ($n > 60$) in the 5' untranslated region (UTR) of *FMR1* gene were linked to fragile X syndrome, a common form of mental retardation (Fu *et al.*, 1991; review-Orr and Zoghbi, 2007). As a result of this mutation, increased DNA and histone methylation levels (i.e. H3K9) and reduced histone

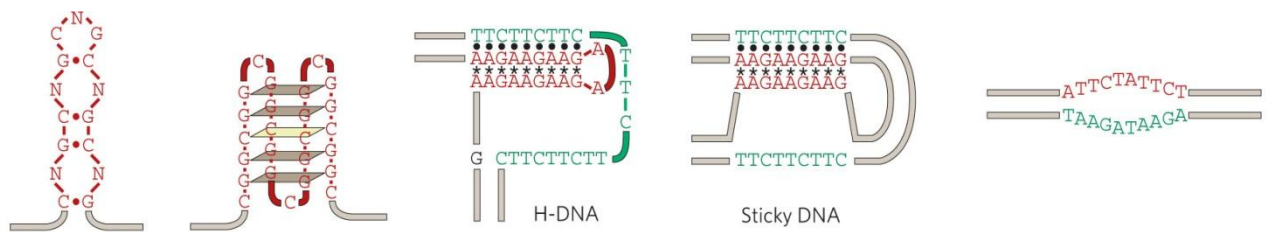
acetylation levels (i.e. H3K9, H4K16) were reported on the transcriptionally silenced *FMR1* gene, which encodes an RNA binding protein that functions in neuronal development (Biacsi *et al.*, 2008; Brown *et al.*, 2001; Hornstra *et al.*, 1993; Sutcliffe *et al.*, 1992). Perhaps the most well-known example of gene silencing induced by triplet repeats occurs in Friedreich's ataxia, which is one of the main research focuses of this thesis. Briefly, Friedreich's ataxia is caused by the intronic (GAA)_n expansion (n>66) (Campuzano *et al.*, 1996) which is associated with decreased transcriptional processivity due to the resultant triplex DNA and increased levels of heterochromatin marks (DNA methylation, H3K9 methylation, HP1 recruitment and loss of H3K4 methylations and histone acetylations) along the *FXN* gene that encodes mitochondrial protein Frataxin [see section 1.9]. In addition to the mechanisms raised so far, more recent studies addressed the possibility of RNAi mediated heterochromatinisation provoked by double stranded RNA structures raised from microsatellites due to bidirectional transcription or secondary structures. These recent findings particularly suggest the importance of RNAi pathways in gene silencing induced by (CNG)_n and (GAA)_n repeats whose antisense transcription seems to be regulated by the chromatin insulator CTCF (Cho *et al.*, 2005; De Biase *et al.*, 2009; Krol *et al.*, 2007; Ladd *et al.*, 2007). Another explanation for the heterochromatin-inducing effect of microsatellites could be the strong preferential nucleosome affinity due to reduced DNA flexibility and curvature, as shown for (CTG)_n repeats (Wang *et al.*, 1994; Wang and Griffith, 1995). Interestingly, the fact that hCD2 gene expression [see section 1.5.6] variegates in the presence of (CTG)_n and (GAA)_n repeats despite its euchromatic location strengthens the hypothesis that minisatellite-induced disease phenotypes could be a result of PEV – like gene expression (Saveliev *et al.*, 2003).

It is also important to note that some repeat elements may cause disease via gain-of-function at RNA or protein level. For example, in myotonic dystrophy (DM -1 and -2); (CTG)_n repeats (n>50) located in the 3'UTR of *DMPK* gene encoding a protein kinase (DM1) or intronic (CCTG)_n repeats (n>75) on *ZNF9* gene encoding a zinc finger protein are known to dysregulate splicing of certain nuclear RNAs by inducing depletion or over-expression of specific RNA-binding proteins, which are overwhelmingly attracted to the secondary RNA structures caused by these repetitive elements (Brook *et al.*, 1992; Kuyumcu-Martinez *et al.*, 2007; Liquori *et al.*, 2001; Miller *et al.*, 2000). Similar mechanisms involving RNA gain-of-function are also known to occur in fragile X tremor syndrome and some forms of spinocerebellar ataxias. These are reviewed elsewhere (review-Brouwer *et al.*, 2009). Lastly, gain-of-function at protein level happens when an expanded triplet microsatellite resides within an exon, where it is interpreted as an amino-acid code. Accordingly, exonic (CAG)_n repeats introduce polyglutamine residues into the encoded protein and (GCN)_n tracts are

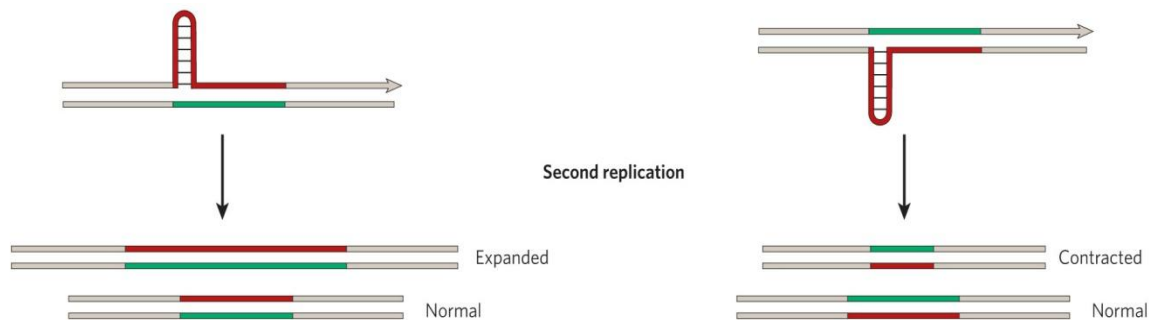
known to create polyalanine mutations (review-Brouwer *et al.*, 2009; review-Mirkin, 2007). These mutations then disrupt the native function of the affected protein and thereby provoke a disease phenotype. A well-known example is Huntington's disease, where the exonic (CAG)_n expansion (n>40) on *IT15* gene results in a stretch of disrupting polyglutamine residues on its protein product; Huntingtin, which is implicated in transcription, transport and signalling via its interacting partners (Brook *et al.*, 1992; review-Gemayel *et al.*, 2010; Rubinsztein *et al.*, 1996).

Figure 1.23

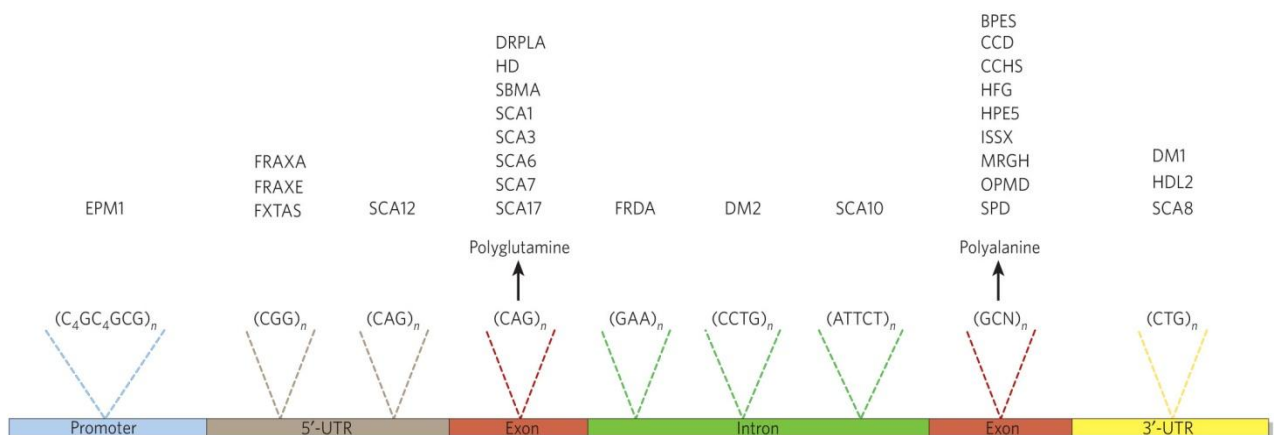
A.



B.



C.



See next page for figure legend.

Figure 1.23 - Unusual DNA structures formed by mini- / micro- satellites, repeat expansion by strand slippage and neurological diseases associated with repeat elements. [A] Unusual DNA structures formed by expandable mini- and microsatellites. Structure-prone strand is shown in red, complementary strand in green and flanking DNA in beige. (CNG)_n repeats can form imperfect hairpins: (CGG)_n, (CCG)_n and (CGCG₄CG₄)_n repeats can induce G- quartets introducing tetrahelical structures. Moreover, (GAA)_n repeats may inhibit reverse Hoogsteen pairing (indicated with asterisks) and adapt triplex structures known as H-DNA or sticky DNA. Finally, DNA unwinding elements could be formed by (ATTCT)_n•(AGATT)_n repeats. **[B] Repeat expansion by strand-slippage.** Unusual DNA conformations presented in (B) could give rise to repeat expansions. For example, formation of a repetitive hairpin on the nascent strand could introduce an expansion mutation after the second replication (left panel). On the other hand, if the hairpin is on the template strand, this could be resulted in a contraction (right panel). **[C] Neurological diseases associated with expanded repeat elements.** Repeat expansions that take place within a gene can cause disease by dysregulating the expression levels of the subject gene or introduce RNA or protein gain-of-functions.

[BPES: blepharophimosis, ptosis and epicanthus inversus, CCD: cleidocranial dysplasia, CCHS: congenital central hypoventilation syndrome, DM: myotonic dystrophy, DRPLA: dentatorubral–pallidoluysian atrophy, EPM1: progressive myoclonic epilepsy 1, FRAXA: fragile X syndrome, FRAXE: fragile X mental retardation associated with *FRAXE* site, FRDA: Friedreich's ataxia, FXTAS: fragile X tremor and ataxia syndrome, HD: Huntington's disease, HDL2: Huntington's-disease-like 2, HFG: hand–foot–genital syndrome, HPE5: holoprosencephaly 5, ISSX: X-linked infantile spasm syndrome, MRGH: mental retardation with isolated growth hormone deficiency, OPMD: oculopharyngeal muscular dystrophy, SBMA: spinal and bulbar muscular atrophy, SCA: spinocerebellar ataxia, SPD: synpolydactyly. B, C and D adapted from (review-Mirkin, 2007)]

1.9 Objectives

The primary aim of this Ph.D. study was to further understand the mechanisms whereby heterochromatin influences gene expression. For this purpose, the *FXN* gene, where abnormal heterochromatinisation is implicated in the causation of the neurological disease Friedreich's ataxia, was studied as a model locus. It was reasoned that understanding the molecular mechanisms behind pathological *FXN* silencing could have general implications regarding heterochromatin-mediated gene silencing. Importantly, it was hoped that these studies might enable the development of a novel therapeutic approach against this currently untreatable disorder.

Another aim of this thesis was to investigate how heterochromatin influences sexual dimorphisms, which are known to result in sex bias in several diseases. Here, the effect of sex chromosome complement on heterochromatin-mediated gene regulation was of special interest in the context of repetitive DNA.

CHAPTER 2
HETEROCHROMATIN EFFECTS IN FRIEDREICH'S ATAXIA

2.1 Introduction

2.1.1 General information

Friedreich's ataxia (FRDA) is the most common autosomal recessive neurodegenerative disorder with an incidence of 1/50,000 in the caucasian population (Campanella *et al.*, 1980; Friedreich, 1863; Koeppen, 2011; Leone *et al.*, 1990; Winter *et al.*, 1981). It is characterised clinically by progressive gait and limb ataxia, dysarthria, areflexia, loss of vibratory and position sense, and distal extremity weakness. Additional features include hypertrophic cardiomyopathy, scoliosis and diabetes (review-Durr *et al.*, 1996). Neurologically, FRDA patients exhibit pathological abnormalities in the spinocerebellar tracts, dorsal columns, cerebellum and medulla (review-Koeppen, 2011). Finally, most fatalities occur because of cardiac dysfunction (Hewer, 1968). Disease onset takes place around puberty with a life expectancy of 40-50 years (review-Schulz *et al.*, 2009). This incurable condition is a result of the depletion or malfunction of the Frataxin protein encoded by the *FXN* gene. In the majority of cases, it was linked to a pathological expansion of polymorphic microsatellite (GAA)_n repeats within the first intron of the *FXN* gene causing its silencing (Campuzano *et al.*, 1996; review-Pandolfo, 2001). The size associated with FRDA ranges from 66 to 1700, being most prevalent around 600-900 repeats (Campuzano *et al.*, 1996; Cossee *et al.*, 1997; Durr *et al.*, 1996). Rarely (in ~2% of cases) missense, nonsense or splice-site point mutations were reported (Bidichandani *et al.*, 1997; Cossee *et al.*, 1999; review-Pandolfo, 2001).

2.1.2 *FXN* gene

As illustrated in figure 2.1, the human *FXN* gene resides on chromosome 9 (9q21.11) on the positive strand and contains 5 exons and three putative CpG islands [(review-Pandolfo, 2008b); <http://cpgislands.usc.edu/>]. Minor splice variants exist with unknown functional significance. Polymorphic (GAA)_n repeats are found within the first intron of the human *FXN* inside an Alu element, that is absent in rodents (Campuzano *et al.*, 1996; Cossee *et al.*, 1997; Greene *et al.*, 2007). Next to the (GAA)_n repeats, a polymorphic mononucleotide tract of adenines (poly A) was identified (Monticelli *et al.*, 2004). Transcription is known to start 221 bp upstream (TSS) of the translation start site ATG (Campuzano *et al.*, 1996). However, a recent *in vitro* study suggests an alternative transcription start site located 159 bp downstream of the original TSS (Kumari *et al.*, 2011). *FXN* is expressed in all cells at a low level but relatively higher in tissues with high metabolic rate. These include liver, kidney, brown fat and heart. Significant levels of expression were also detected in spinal cord, cerebellum and forebrain (Al-Mahdawi *et al.*, 2006; Campuzano *et al.*, 1997; Koutnikova *et al.*, 1997).

Figure 2.1

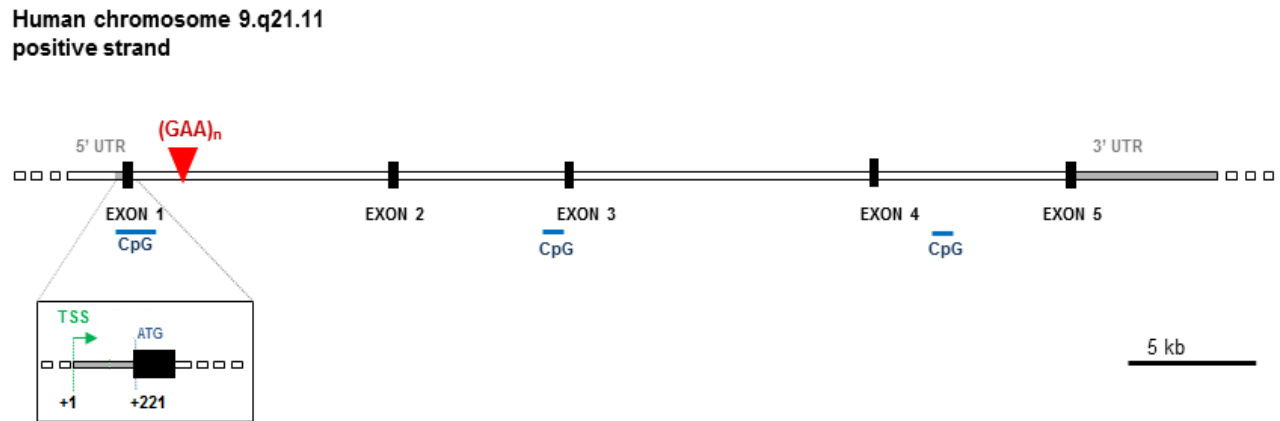


Figure 2.1 – The human *FXN* gene. *Frataxin* gene consists of five exons and resides on the positive strand of the human chromosome 9 (q21.11). The transcription start site (TSS) is located 221bp upstream of the ATG of the first exon (NCBI-Gene). The *FXN* gene has three putative CpG islands (CpG island searcher; <http://cpgislands.usc.edu/>). Polymorphic (GAA)_n repeats are located in the first intron ~1300 bp further from the first exon.

2.1.3 Frataxin protein

Frataxin is a 210 amino-acid long, evolutionarily conserved globular protein, whose N terminal tail comprises a mitochondrial import signal (Campuzano *et al.*, 1996; Gibson *et al.*, 1996; review-Pandolfo and Pastore, 2009). Indeed, it undergoes functional maturation inside the mitochondria (Branda *et al.*, 1999; Koutnikova *et al.*, 1998), where functions as an allosteric component of a multimeric enzyme complex (SDUF: NSIF1, ISD11, ISU2 and FXN) that performs the biogenesis of iron-sulphur clusters (ISCs) (Bridwell-Rabb *et al.*, 2012; Bridwell-Rabb *et al.*, 2011; Tsai and Barondeau, 2010). Here, FXN binding favours the conformational changes needed for the catalytical activity of the SDUF complex. ISCs serve as prosthetic groups for a series of enzymes with various functions including energy metabolism (aconitases and respiratory chain complexes), iron metabolism (iron responsive protein I, ferrochelatase), purine synthesis and DNA repair (Huynen *et al.*, 2001; review-Pandolfo and Pastore, 2009). Similarly, Frataxin is involved in the final step of heme biosynthesis by interacting with ferrochelatase (Foury and Cazzalini, 1997). Moreover, it was also postulated to play a role in the response to oxidative stress by reducing reactive oxygen species (ROS) through binding to Fe^{2+} ions directly, converting them to Fe^{3+} and holding the attached metal in the bioavailable form; preventing the occurrence of the 'Fenton reaction' that produces highly toxic $\text{OH}\cdot$ radical (Babcock *et al.*, 1997; Park *et al.*, 2002; Park *et al.*, 2003).

Frataxin deficient cells therefore exhibited iron accumulation and were shown to be particularly sensitive to oxidative stress (Al-Mahdawi *et al.*, 2006; Babcock *et al.*, 1997; Chen *et al.*, 2004; Foury and Cazzalini, 1997; Jiralerspong *et al.*, 2001; Wong *et al.*, 1999). However, this could also be a secondary manifestation as complete Frataxin deficiency led to embryonic lethality without evidence of iron accumulation in mice (Cossee *et al.*, 2000). In addition, Palomo *et al.* (2011) recently reported that short hairpin RNA (shRNA) mediated Frataxin depletion caused upregulation of the proapoptotic factors P53, PUMA and BAX as well as activation of caspase-3; resulting in large-scale cell death of differentiated neuron-like cells. Frataxin protein levels correspond well with the mRNA levels in peripheral blood mononuclear cells (Sacca *et al.*, 2011). Still, some studies based on mouse models reported that mRNA levels do not always correspond well with the protein levels (Al-Mahdawi *et al.*, 2006; Sandi *et al.*, 2011). Notably, Frataxin is considered to be thermodynamically stable (half-life of ~50 hours) under normal physiological conditions and is regulated by the ubiquitin-proteasome system (Li *et al.*, 2008; review-Pandolfo and Pastore, 2009; Rufini *et al.*, 2011). Although these findings revealed some clues about the Frataxin protein, its exact function and the reason why it is essential for embryonic development still remain unknown (review-Pandolfo, 2008b).

2.1.4 Experimental models of Friedreich's ataxia

Most of the FRDA research is initially conducted on primary lymphocytes or fibroblasts obtained from patients. Also, patient derived lymphoblastoid cells immortalised by Epstein-Barr virus (EBV) transformation are among popular choices for FRDA research (Coriell Cell Repositories, <http://ccr.coriell.org/>). These cell lines provide a substantial source for many protocols that need high cell numbers (e.g. ChIP), despite the fact that they start to show considerable genetic instability after ~30 generations (Mohyuddin *et al.*, 2004). In addition to this, recently developed induced pluripotent stem cell (iPS) technology (Okita *et al.*, 2007; Takahashi *et al.*, 2007; Takahashi and Yamanaka, 2006) allowed scientists to generate differentiated neuron-like cells derived from FRDA patients (Ku *et al.*, 2010; Liu *et al.*, 2011). Undoubtedly, mouse models provide a valuable tool to study disease mechanism and potential treatments. Murine FRDA models either have the endogenous *Fxn* gene knocked out and are rescued from embryonic lethality by a human *FXN* transgene that carries the pathological (GAA)_n repeat expansion; or have the GAA-repeat expansion inserted into the mouse *Fxn* gene. Among these, KIKI knock-in mouse in which the mouse is homozygous for the GAA-repeat knock-in and human *FXN* YAC transgenic mice were used in numerous studies (review-Puccio, 2009).

The KIKI mouse has a (GAA)₂₃₀ insertion that is not directly comparable to the intronic location in humans and it failed to exhibit an FRDA phenotype of motor deficits and iron overload (Miranda *et al.*, 2002). On the other hand, YAC transgenic mice (subtypes: YG22 and YG8, both C57BL/6J background) exhibited a mild FRDA phenotype characterised by pathological abnormalities and neurophysiological deficits as well as increased sensitivity to oxidative stress (Al-Mahdawi *et al.*, 2006). The YG8 mouse, which has two copies of the human *FXN* transgene with 90+190 repeats, is the FRDA animal model used in this thesis (Al-Mahdawi *et al.*, 2004). YG8 mice exhibit decreased levels of Frataxin protein (42% in cerebellum, 25% in heart and 10% in skeletal muscle) compared to wild type animals (Al-Mahdawi *et al.*, 2006). Both expansion and contraction mutations of (GAA)_n were reported for these mice in the progeny and somatic tissues. Somatic instability seems to be proportional to age (starting after 2 months) particularly in the cerebellum and dorsal root ganglia where the tendency is towards expansion (Al-Mahdawi *et al.*, 2004).

Apart from these, mice with tissue specific Frataxin deficiency (Puccio *et al.*, 2001) and mice carrying an EGFP (enhanced green fluorescent protein) reporter gene fused with the human *FXN* gene (Sarsero *et al.*, 2005) also provide useful tools for FRDA research.

2.1.5 Physiological regulation of *FXN*

Regulatory sequences for normal human *FXN* regulation were reported to be contained within an extended region of 1034 bp upstream and 100 bp downstream of TSS as shown by reporter *FXN* – luciferase constructs in mouse skeletal cells (Greene *et al.*, 2005). The 100bp downstream region, which showed the highest effect on gene expression according to Greene *et al.*, lacks a TATA box but contains Inr/DPE like elements [see general introduction 1.6.2]. Although it is likely that *FXN* expression is controlled by such Inr/DPE elements downstream of the TSS, the same study demonstrated that deletion of these did not significantly disrupt the expression of the gene. Therefore, human *FXN* can be classified as an unusual gene among mammals in terms of the core-promoter function. Interestingly, Greene *et al.* also revealed that the regulatory region extending to 100 bp downstream of TSS contains an ancient remnant of LINE (L2) transposon, whose deletion seems to impair *FXN* expression significantly. Moreover, traces of MIR and Alu elements were also reported to be present upstream of TSS (Greene *et al.*, 2005).

The physiological regulation of *FXN* expression is largely dependent on iron, as experimentally induced depletion of iron resulted in decreased *Frataxin* mRNA and protein levels in FRDA patient lymphoblasts and fibroblasts (Li *et al.*, 2008). This could be one explanation for the downregulation of the *FXN* gene in disease since FRDA is associated with iron accumulation in mitochondria and iron depletion in other cellular compartments (review-Li *et al.*, 2008; Rouault and Tong, 2005). Furthermore, the region between TSS and the ATG was found to be bound by serum response factor 2 (SRF2) and transcription factor AP2 (TFAP2) (Li *et al.*, 2010b). Also, a minimal region of 17 bp, which is located 4.9 kb upstream of the first exon, has recently been identified using *FXN*-reporter constructs and bioinformatic approaches (Puspasari *et al.*, 2011). Although there is no direct evidence yet, this site is potentially regulated by the transcription factor OCT1. Finally, there seems to be a link between the peroxisome proliferator activated receptor gamma (PPAR γ) pathway and *FXN* regulation as low Frataxin levels were associated with decreased levels of PPAR γ coactivator α (PGC1 α) and a PPAR γ agonist upregulates *FXN* in primary FRDA fibroblasts (Coppola *et al.*, 2009; Marmolino *et al.*, 2009; Marmolino *et al.*, 2010). PPAR γ is a nuclear receptor activated by fatty acids and is a key regulator of mitochondrial biogenesis as well as reactive oxygen species (ROS) metabolism (review-Kelly and Scarpulla, 2004; Wu *et al.*, 1999). Importantly, PPAR γ function was implicated in diabetes and this may explain why diabetes is within the clinical frame of FRDA (Coppola *et al.*, 2009; review-Sugden *et al.*, 2010).

2.1.6 (GAA)_n repeat expansion and dysregulation of *FXN* in disease

Normal variation in *FXN* expression levels are usually less than 20% amongst healthy individuals (Boehm *et al.*, 2011; Sacca *et al.*, 2011). In the case of (GAA)_n repeat expansion (>66), Frataxin levels both at the protein and mRNA levels usually drop below ~30% (lowest reported 5%) of healthy levels and the disease phenotype occurs. On the other hand, heterozygous asymptomatic carriers typically express ~50% of Frataxin compared to healthy non-carriers (Boehm *et al.*, 2011; Pianese *et al.*, 2004; Sacca *et al.*, 2011). (GAA)_n repeats exhibit instability, not only when being transmitted from parent to child but also early during development and in somatic cells throughout life (Al-Mahdawi *et al.*, 2004; De Biase *et al.*, 2007b; Montermini *et al.*, 1997b; Montermini *et al.*, 1997c; Pianese *et al.*, 1997). This instability involves both contractions and expansions; however, a tendency to accumulate expansions was detected in the dorsal root ganglia and cerebellum (Al-Mahdawi *et al.*, 2004; Clark *et al.*, 2007; De Biase *et al.*, 2007a). In general, the size of (GAA)_n correlates well with the disease progression (Filla *et al.*, 1996; Monros *et al.*, 1997). It is also noteworthy that the disease severity correlates negatively with the age of onset (Bhidayasiri *et al.*, 2005; Montermini *et al.*, 1997a). Notwithstanding though, some patients (particularly obvious in the Acadian population) have a late onset accompanied by mild symptoms despite having a considerably long repeat size (Montermini *et al.*, 1997a; Montermini *et al.*, 1997c). This suggests that there may be mechanisms in addition to differences in genetic background (e.g. epigenetic) that may participate in the dysregulation of *FXN* expression.

In order to understand the mechanisms behind the pathologic (GAA)_n expansions and transcriptional silencing, one needs to have an insight into the molecular nature of this polymorphic microsatellite repeat. Poly (GAA)_n tracts are known to form non-B DNA conformations by forming intramolecular triple-helix structures due to their mirror repeat pattern that contains purines (R) on one strand and pyrimidines (Y) on the other (review-Wells, 2008). Furthermore, RRY triplexes can also adopt higher order structures under the influence of negative supercoiling [H DNA and sticky DNA; see figure 1.23.A] (Gacy *et al.*, 1998; Sakamoto *et al.*, 1999; review-Wells, 2008). More recently, transcription dependent RNA-DNA hybrids (R-loops) were reported (Grabczyk *et al.*, 2007; McIvor *et al.*, 2010). Notably, studies addressing these unusual conformations were predominantly performed either *in vitro* or in *Escherichia coli* by using plasmids and reporter gene constructs. Therefore, whether these actually take place *in vivo* at the endogenous *FXN* locus needs to be addressed by further experiments (review-Pandolfo, 2008b; review-Schmucker and Puccio, 2010; review-Wells, 2008).

Consistent with general microsatellite instability mechanisms explained in the general introduction [1.8.3.3], (GAA)_n instability was shown to be potentially triggered by replication mediated strand slippage and replication fork stalling in a range of studies (Krasilnikova and Mirkin, 2004; Pollard *et al.*, 2004; Rindler *et al.*, 2006; Shishkin *et al.*, 2009). Expanded (GAA)_n were shown to be stabilised by mismatch repair proteins (particularly MSH2) both in *E. coli* and in human iPS cells (Bourn *et al.*, 2009; Ku *et al.*, 2010). Moreover, a recombination hot spot activity arises from (GAA)_n tracts and this could be another factor contributing to the repeat's instability; as shown in *E. coli* (Napierala *et al.*, 2004; Pollard *et al.*, 2007). Finally, transcription influences (GAA)_n instability but whether it creates a bias for expansion or contraction is not exactly clear (Ditch *et al.*, 2009; Soragni *et al.*, 2008). Although the mechanisms remain elusive, interplays between R-loops and the replication machinery seem to operate in concert to generate different patterns of (GAA)_n instability in mammalian cells, providing a potential explanation for distinct instability tendencies (i.e. expansion/contraction) in different tissues (Grabczyk *et al.*, 2007; McIvor *et al.*, 2010; Rindler and Bidichandani, 2011).

There are two models for explaining the transcriptional silencing of *FXN* in disease. Accordingly, the transcriptional silencing could be either a result of direct physical blockage effect on transcription progress because of the above-explained unusual DNA conformations or a chromatin mediated silencing. These models are not mutually exclusive (review-Schmucker and Puccio, 2010). *In vitro* models and studies with yeast and bacteria concluded that expanded (GAA)_n repeats inhibit transcription in a size and orientation dependent manner based on transcription assays, RNase protection assays and northern blots (Bidichandani *et al.*, 1998; Grabczyk and Usdin, 2000b; Ohshima *et al.*, 1998; Sakamoto *et al.*, 1999; Sakamoto *et al.*, 2001b). Consistently, patient EBV-lymphoblastoid cell lines exhibited transcriptional defects on *FXN* (Kim *et al.*, 2011a; Kumari *et al.*, 2011; Punga and Buhler, 2010). Punga *et al.* reported that transcription happens at a much lower rate in FRDA compared to healthy cells based on RNA kinetics experiments which measured synthesis of *FXN* mRNA. They also reported that the half-life of *FXN* mRNA (~11 hours) was not affected in FRDA cell lines implying that dysregulation does not take place post-transcriptionally. Although transcriptional defects were shown, the character and mechanisms behind these negative effects on *FXN* remain largely to be illuminated.

Transcriptional silencing of *FXN* could also be explained by the fact that (GAA)_n repeats induce heterochromatinisation. The phenomenon stemmed from experiments with the hCD2 reporter gene in transgenic mice [see introduction 1.5.6] that is repressed in a PEV-like manner in the presence of a pathological (GAA)_n repeat expansion (Saveliev *et al.*, 2003).

Furthermore, nuclei double-stained with antibodies against centromeric- and triplex- (GAA)_n tracts revealed a close spatial association of both signals in human cells, suggesting the possibility of chromatin organisation via DNA triplexes (Ohno *et al.*, 2002). Consistently, increased H3K9 (-di and -tri) methylation levels upstream of (GAA)_n and decreased histone acetylation levels (H3K14, H4K5, H4K8, H4K12 and H4K16) both at the promoter and gene body were reported in FRDA lymphoblastoid cells (Greene *et al.*, 2007; Herman *et al.*, 2006; Kim *et al.*, 2011a; Kumari *et al.*, 2011; Punga and Buhler, 2010; Soragni *et al.*, 2008) as well as in human and mouse brain tissues (Al-Mahdawi *et al.*, 2008). Notably, Kim *et al.* also reported elevated H3K27me3 and H4K20me3 levels upstream of expanded (GAA)_n. Which HDACs are involved in the deacetylation of histones within the context of FRDA is not clear yet; however, some studies suggest a possible role for HDAC3 based on chemical inhibitions and photoaffinity labelling assays (Herman *et al.*, 2006; Xu *et al.*, 2009a).

In addition to histone modifications, hypermethylation of CpG islands upstream of a pathological (GAA)_n expansion followed by a downstream hypomethylation were reported (Al-Mahdawi *et al.*, 2008; Greene *et al.*, 2007). This shift could be explained by the known position of (GAA)_n within an Alu sequence, which can attract methylation and cause its bi-directional spread (Al-Mahdawi *et al.*, 2008; Graff *et al.*, 1997). Also, one of the methylation sites overlaps with an E-box, which provides a binding site for basic-helix-loop-helix transcription factors (Greene *et al.*, 2007). On the contrary though, another study reported no difference between healthy and FRDA cells in DNA methylation levels at the 5' UTR (De Biase *et al.*, 2009). They also report HP1 -α and -γ enrichment accompanied by H3K9me3 and H3K27me3 marks at the 5' UTR in the case of FRDA (De Biase *et al.*, 2009). This region overlaps with the +1 nucleosome, which is an essential regulator of transcription (Jiang and Pugh, 2009). Intriguingly, De Biase *et al.* also reported antisense transcription (*FXN* antisense transcript 1: FAST-1) that is 236 nt long in the reverse direction starting from the +136 position relative to TSS. Heterochromatinisation of +1 nucleosome and antisense transcription seem to be elevated in FRDA with the depletion of CTCF binding between +154 and +173. Overall, studies described here indicated that (GAA)_n repeats induce heterochromatin mediated silencing of *FXN* in the case of FRDA; however, how heterochromatin is recruited in the first place and the mechanistic aspects of it has not been elucidated yet.

2.1.7 Therapeutic strategies for Friedreich's ataxia

FRDA is still incurable; however, several promising therapeutic strategies have been developed. These are either addressing the symptoms and course of the disease or attempting to upregulate the *FXN* gene directly.

Because ROS accumulation and oxidative damage is a characteristic of FRDA; coenzyme Q₁₀ (CoQ₁₀) and vitamin E were considered as potential treatments to alleviate symptoms of the disease. Vitamin E is a cellular lipid-soluble antioxidant and CoQ₁₀ is an electron carrier in the respiratory chain and reduces the oxidised form of vitamin E (review-Ernster and Dallner, 1995). Indeed, a combination treatment of both vitamin E and CoQ₁₀ improved cardiac and skeletal muscle bioenergetics in FRDA patients even though there was no significant effect on cardiomyopathy (Hart *et al.*, 2005; review-Pandolfo, 2008a). Also, idebenone (a short chain analogue of CoQ₁₀) suggested to have significant benefits in deterioration of neurological conditions as well as cardiac hypertrophy in FRDA patients (Buyse *et al.*, 2003; Mariotti *et al.*, 2003; Rustin *et al.*, 1999a). Still no improvement on cardiac function was reported in long-term follow up with idebenone treatment (Ribai *et al.*, 2007). In addition to these, iron chelators (e.g. deferoxamine, deferiprone) were examined in order to address excess iron-accumulation in FRDA (Boddaert *et al.*, 2007; Rustin *et al.*, 1999b). However, the results for these were not promising either due to confounding effects (review-Santos *et al.*, 2010).

In order to replace decreased Frataxin, several gene therapy approaches were studied. Recombinant adeno-associated and lentiviral vectors, as well as herpes simplex virus type 1 (HSV1) amplicon vectors expressing *FXN* cDNA were shown to partially correct sensitivity to oxidative stress in FRDA fibroblasts or animal models (Fleming *et al.*, 2005a; Gomez-Sebastian *et al.*, 2007; Lim *et al.*, 2007). Still, these require a significant amount of medical research in order to set up *FXN* gene therapy in clinics. In search for readily applicable therapies, small molecules that target the *FXN* gene, hemin (an iron containing compound synthesised in mitochondria), butyric acid (histone deacetylase inhibitor) and erythropoietin (a glycoprotein hormone that involves in erythropoiesis and protein stabilisation) are amongst the first ones shown to upregulate *FXN*. Less than 1.5 fold increase was reported with hemin and butyric acid although the mechanism of action is not clear (Sarsero *et al.*, 2005; Sarsero *et al.*, 2003). On the other hand, recombinant erythropoietin is thought to be involved in the stabilisation of Frataxin protein since it did not affect mRNA levels (Acquaviva *et al.*, 2008; Sturm *et al.*, 2005). Interestingly, a recent report revealed PPAR γ agonist Azelaoyl-PAF as a *FXN* upregulating factor (~ 2-fold level both at protein and mRNA level); however, this laboratory standard compound is not yet considered as a potential treatment

(Marmolino *et al.*, 2009). Drug screens also revealed some small molecules (e.g. cisplatin, 3-nitropropionic acid, distamycin, pentamidine) with potential effects on the *FXN* gene although they are not considered to be of therapeutic interest (review-Santos *et al.*, 2010). Furthermore, several molecules have been developed to target the non-B DNA conformation of (GAA)_n repeats. Polyamides increased *FXN* levels in FRDA lymphoblastoid cell lines by specifically binding to (GAA)_n tracts (Burnett *et al.*, 2006). Also, oligo-deoxyribonucleotides designed to block triplex formation enhanced *FXN* transcription *in vitro* (Grabczyk and Usdin, 2000a).

Because FRDA has been thought to be caused by the heterochromatinisation of the *FXN* gene, studies addressing this issue inspired by the field of epigenetics took particular attention in the last years. The potential of HDAC inhibitors in preventing heterochromatinisation made them good candidates for FRDA therapeutics. This could be achieved by shifting the equilibrium towards acetylation and thereby reducing the chances of subsequent methylation (review-Festenstein, 2006). Interestingly, none of the classic HDAC inhibitors such as TSA or SAHA could upregulate *FXN* (Herman *et al.*, 2006; Xu *et al.*, 2009a). On the other hand, BML-210 (*N*¹-[2-aminophenyl]-*N*⁸-phenyloctanediamide) and its derivatives could upregulate the gene in FRDA primary lymphocytes and cell lines significantly [in a range of 1.2-3.5 fold] (Herman *et al.*, 2006). These pimelic diphenylamide molecules seem to act specifically on HDAC class I and particularly on HDAC3 (Chou *et al.*, 2008; Xu *et al.*, 2009a). Importantly, they were shown to increase Frataxin protein and mRNA levels in the nerve and heart tissues of KIKI (GAA)₂₃₀ and YG8 mouse models as well as increasing histone acetylation marks (H3K14, H4K5, H4K8, H4K16) with no obvious effect on H3K9 methylation (Rai *et al.*, 2010; Rai *et al.*, 2008; Sandi *et al.*, 2011).

2.1.8 Hypothesis

Although previous studies indicated that pathologically expanded (GAA)_n repeats induce heterochromatin on the *FXN* gene in FRDA, the components of this pathologic heterochromatin and mechanistic impacts behind the transcriptional silencing phenomenon are largely unknown. Here, it is hypothesised that HDACs (i.e. sirtuins) as well as other components of heterochromatin contribute to the establishment of this pathological *FXN* silencing. Therefore, inhibiting their action would upregulate the gene and could provide a therapeutic approach that directly addresses the cause of FRDA.

2.2 Results

2.2.1 Heterochromatin is spreading from expanded (GAA)_n repeats on *FXN*

Although (GAA)_n repeats had been thought to induce heterochromatin based on the finding of hCD2 reporter system (Saveliev *et al.*, 2003), there was virtually no direct evidence on the endogenous *FXN* locus regarding to the heterochromatic structure induced by expanded (GAA)_n repeats, at the time this Ph.D. study started. Therefore, chromatin immunoprecipitation (ChIP) experiments were performed on the *FXN* locus using antibodies against heterochromatic histone marks as well as proteins. Because the critical tissues for FRDA (e.g. cerebellum, spine, heart..etc) are difficult to obtain, experiments were performed on EBV-transformed lymphoblastoid (immature B-type lymphocyte) cell lines. It was also true that for future experiments (e.g. drug treatments), these would be advantageous because of the continuous cell-culture potential. These cell lines were obtained from Coriell Cell Repositories [<http://ccr.coriell.org/>].

Here, a 'normal' cell line (GM14926; 6-34 GAA repeats) derived from a healthy male at the age of 38 was used as a control. Two cell lines were selected for testing differences at the chromatin level between the healthy and disease state. GM15850 was derived from a 13 years old male FRDA patient who manifested the disease at the age of 10. On the other hand, GM16234 was obtained from a 39 year-old male patient where the disease had a late onset that occurred at the age of 20. Importantly both disease cell lines have a similar level of (GAA)_n repeat expansion (GM15850: 650/1030 repeats; GM16234: 580/1030 repeats) [Figure 2.2A]. Therefore, the effects of (GAA)_n repeats were thought to be partially controlled and any difference between the two cell lines might be attributed to epigenetic variations. Cell lines were previously tested by Nadine Rothe for the correct karyotype (Ph.D. thesis-Rothe, 2008). During this Ph.D. study, their *FXN* expression levels were tested at various occasions by synthesising cDNA from the total RNA followed by quantitative real-time PCR (Q-RT-PCR) as well as a lateral-flow dipstick protein assay (Mitosciences). Primary transcript levels were obtained by the analysis of cDNA synthesised with random hexamers and using primers comprising the Intron 3-Exon 4 junction. mRNA levels were analysed using a primer set that amplify the whole mature transcript (Exon1-Exon5) on the cDNA synthesised with oligo(dT). Accordingly, primary transcript levels were ~30% and ~17% in GM15850 and GM16234, respectively compared to the healthy cell line [Figure 2.2B]. Similarly, *FXN* mRNA was found to be ~23% to ~9% in the disease cell lines. Agreeing well with these results, protein levels were found to be ~19% and ~13% relative to the healthy cell line [see supplementary figure S.2]. As established before (Ph.D. thesis-Rothe, 2008), ChIP experiments were performed by focusing on a region that covers upstream of the *FXN*,

5' UTR and includes the first exon and intron as well as the (GAA)_n repeats [Figure 2.2C]. Because repeats are unstable, trying to analyse this region was problematic. However, this was overcome by using a primer set specific to the flanking regions of (GAA)_n. This enabled assessment of the influence of (GAA)_n repeats on the chromatin structure and RNAPII levels at the *FXN* locus. Indeed, robust heterochromatin marks H3K9me3 and H3K27me3 were enriched in the pathologic *FXN* locus in both FRDA cell lines, in contrast to the healthy cell line [Figure 2.3]. Moreover, HP1β levels were higher in the disease cell lines in a similar pattern with its partner histone modification H3K9me3. Interestingly, all heterochromatic signals were particularly enriched immediately upstream or downstream of the pathologic (GAA)_n repeats. In other words, heterochromatin marks seem to spread from the pathologic repeats in either direction. This strongly suggests that (GAA)_n repeats are indeed nucleating heterochromatin on the endogenous *FXN* locus, as also demonstrated by Punga *et al.* (2010). Heterochromatin influence in the disease cell lines seem to decrease within the 5' UTR and Exon1; however, increased levels of HP1β were detected in the Exon 1 of disease cell lines. It is also notable that GM16234 has slightly higher levels of H3K27me3 and HP1β compared to GM15850. This is consistent with the *FXN* repression levels presented in figure 2.1B. Interestingly, the upstream region represented by UpsP1 primer also carries high levels of heterochromatin marks and HP1β, particularly in the disease cell lines. Bearing in mind that this region is 4.5kb away from the (GAA)_n repeats, it is reasonable to expect it to serve as a regulatory element, perhaps by directly interacting with the (GAA)_n repeats.

It was also noted that the healthy cell line displayed a low level of the H3K9me3 mark, consistent with the fact that low levels of H3K9me3 are present at transcriptionally active genes (Barski *et al.*, 2007; Mikkelsen *et al.*, 2007; Vakoc *et al.*, 2006; Wang *et al.*, 2008). Still, the small HP1β peak in the healthy cell line around the unexpanded (GAA)_n raises the possibility that a low level of heterochromatin is present in the healthy *FXN* locus. This might provide, at least in part, an explanation for the fact that *FXN* is expressed at relatively low levels even in healthy individuals (Campuzano *et al.*, 1997; Su *et al.*, 2004).

Interestingly, HP1γ was peaking in all cell lines around the (GAA)_n repeats and -to a greater extent- at the first exon. As opposed to heterochromatic histone marks and HP1β, no significant enrichment was detected in HP1γ levels compared to healthy control. Still, the distribution of HP1γ appeared more diffuse in patients. In this case, it could be speculated that HP1γ is involved in the native regulation of the endogenous *FXN* gene and may contribute to its dysregulation indirectly perhaps by interacting with the other components of extensive heterochromatin in the case of expanded (GAA)_n repeats.

Figure 2.2

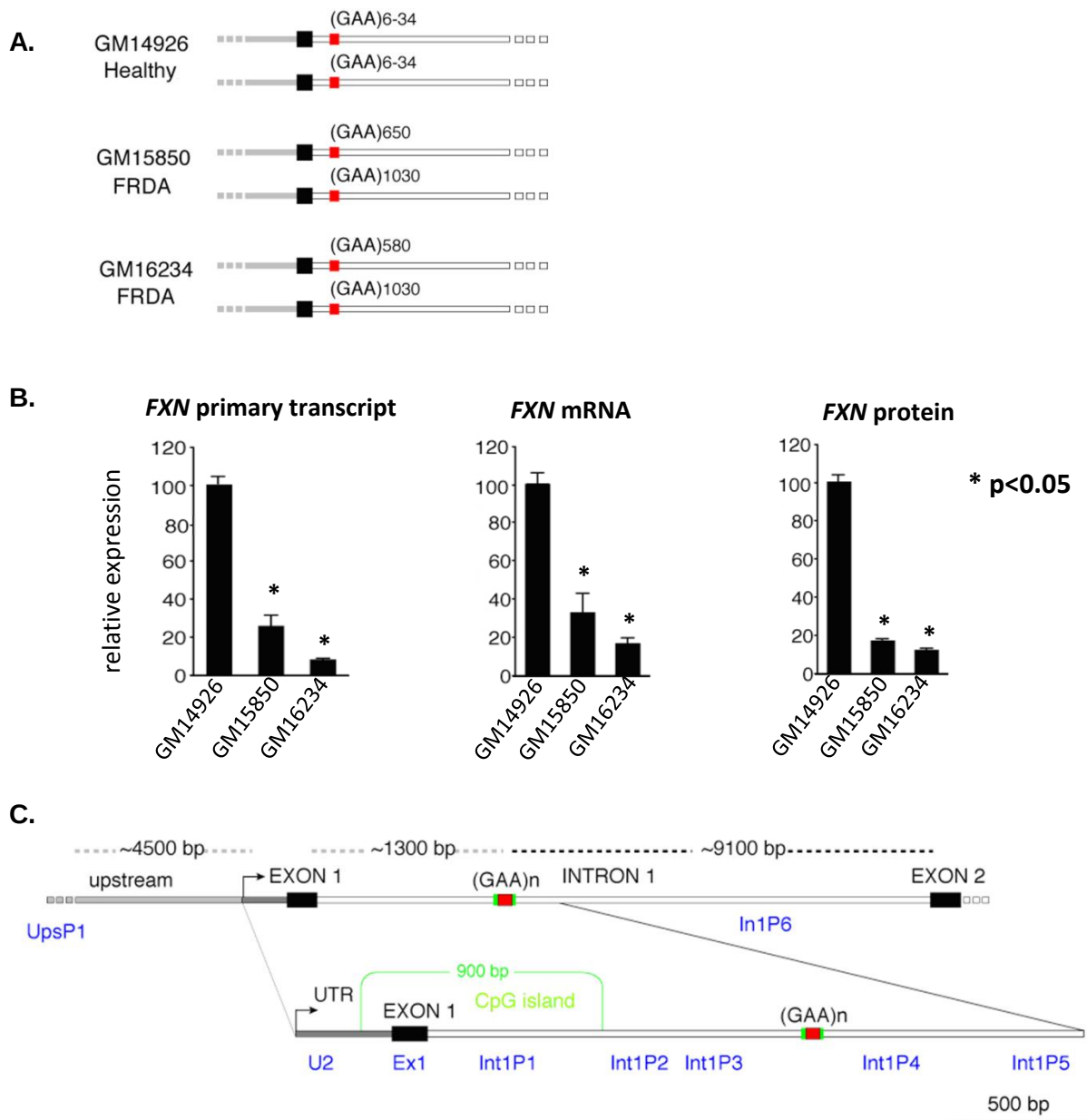


Figure 2.2 – Experimental approach to study gene regulation at the *FXN* locus. [A] Cell lines.

EBV-transformed lymphoblastoid cells derived from a healthy individual and two FRDA patients were used throughout this study. Disease cell lines have about the same level of repeat expansion.

[B] *FXN* expression levels of cell lines. Primary transcripts and mRNA levels of FRDA cells relative to healthy control are shown as detected by Q-RT-PCR after normalisation against β -actin expression. Protein levels relative to the healthy cell line were analysed by the Mitosciences dipstick assay and largely agree with the transcript analysis [*p<0.05 ; student's t-test in comparison to the healthy cell line. Error bars: SEM, n=3]. [C] Primers used throughout ChIP experiments. 9 primers focusing both upstream and downstream of (GAA)_n repeats were used to analyse the influence of (GAA)_n repeats on the *FXN* locus.

Figure 2.3

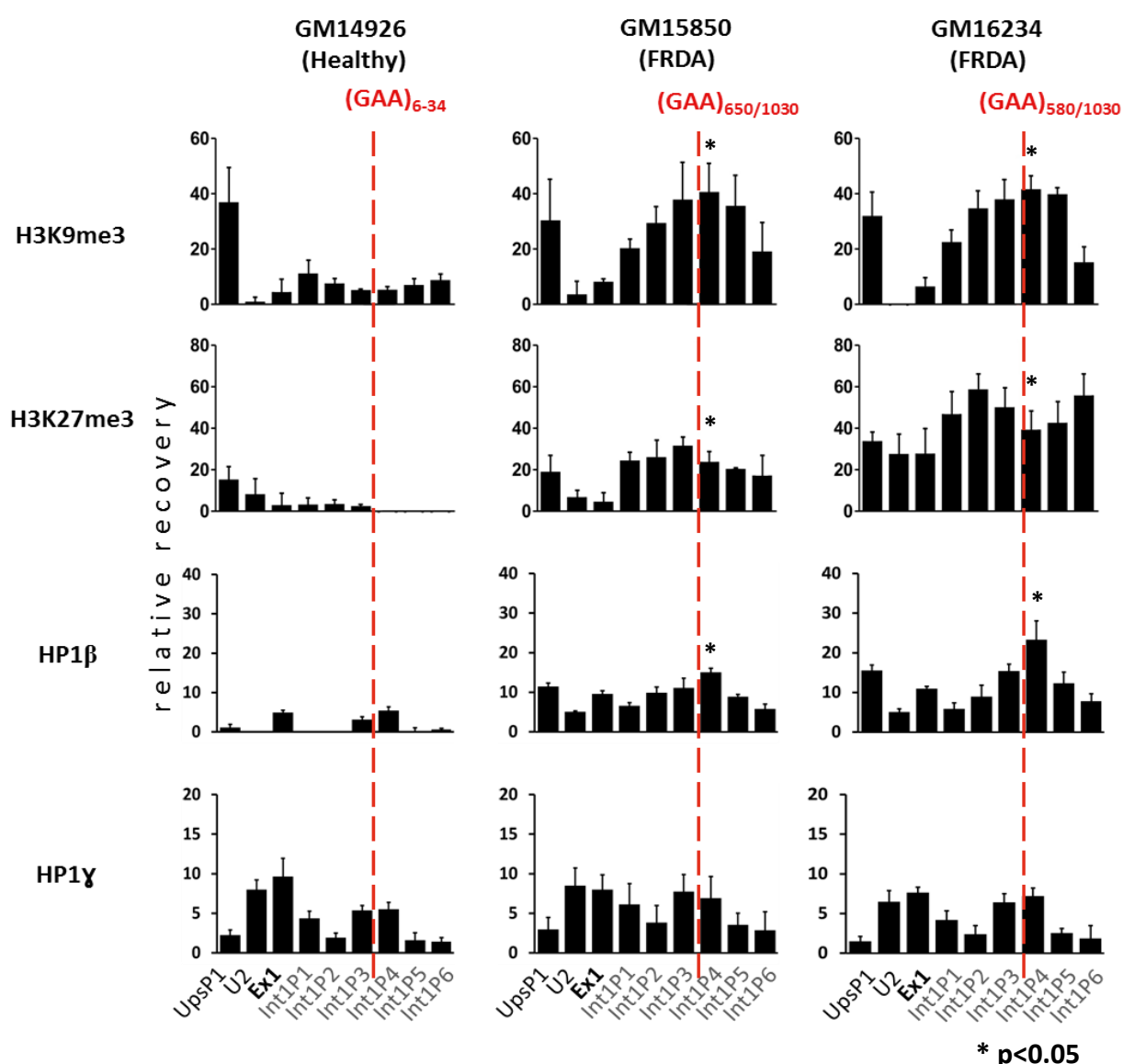


Figure 2.3 – Heterochromatin marks and HP1 binding at the first part of *FXN* locus.

ChIP was performed on the healthy and FRDA cell lines using highly specific antibodies against the robust heterochromatin marks H3K9me3 and H3K27me3 as well as heterochromatin proteins HP1β and HP1γ. Repressive histone marks and HP1β were enriched in the disease cell lines, particularly around the (GAA)_n repeats. No significant difference was observed for HP1γ binding between the healthy and FRDA cell lines. [ChIP signal was shown as recovery percentage relative to H3. * Student's t-test for testing the difference in the ChIP signal from the (GAA)_n flanking Int1P4 region between the healthy and FRDA cells. Error bars: SEM, n=3.]

2.2.2 Chromatin insulator CTCF and cohesin on the *FXN* locus

Having revealed the enrichment of heterochromatin factors on the pathologic *FXN* locus, it was wondered whether chromatin insulators are involved in the regulation of *FXN*. As explained in general introduction 1.5.4, some insulator elements act as a barrier against the spreading of heterochromatin. In order to see if such factors are present at the *FXN* locus, ChIP was performed on the healthy and FRDA cell lines against the prototypic chromatin insulator protein CTCF. Both in healthy and FRDA cells, CTCF was shown to be recruited particularly at Ex1 [Figure 2.4]. A slight decrease for CTCF binding on Ex1 was noted in the GM15850 (FRDA) cells but it this was not statistically significant. Notably, De Biease *et al.* (2009) reported significant depletion of CTCF binding in FRDA cells in a region very close to the sequence amplified by the Ex1 primer set used in this study.

Interestingly, CTCF is known to exert both barrier (Barski *et al.*, 2007; Bartkuhn *et al.*, 2009; Bell *et al.*, 1999; Cuddapah *et al.*, 2009; West *et al.*, 2004a) and enhancer-blocking activities (Chung *et al.*, 1997; Chung *et al.*, 1993). It is also known to co-operate with cohesin rings in mammalian cells in order to exert chromatin insulation functions [see general introduction 1.5.4] (Degner *et al.*, 2009; Seitan *et al.*, 2011; Wendt *et al.*, 2008). SMC3 and RAD21 subunits of the cohesin ring were shown to be essential for this co-operation to take place (Haering *et al.*, 2008). Therefore, the binding of these proteins was also tested by ChIP and a binding pattern similar to that obtained for CTCF was detected. CTCF and cohesin binding on Ex1 could provide a protective mechanism preventing the spreading of (GAA)_n induced heterochromatin into Ex1, where low levels of heterochromatic marks [Figure 2.3] and high levels of RNAPII were detected [Figure 2.9]. In addition, both CTCF and cohesin particles were shown to be bound slightly higher in the intronic region of FRDA cells compared to healthy. This will be discussed further in discussion 2.3.1.

Figure 2.4

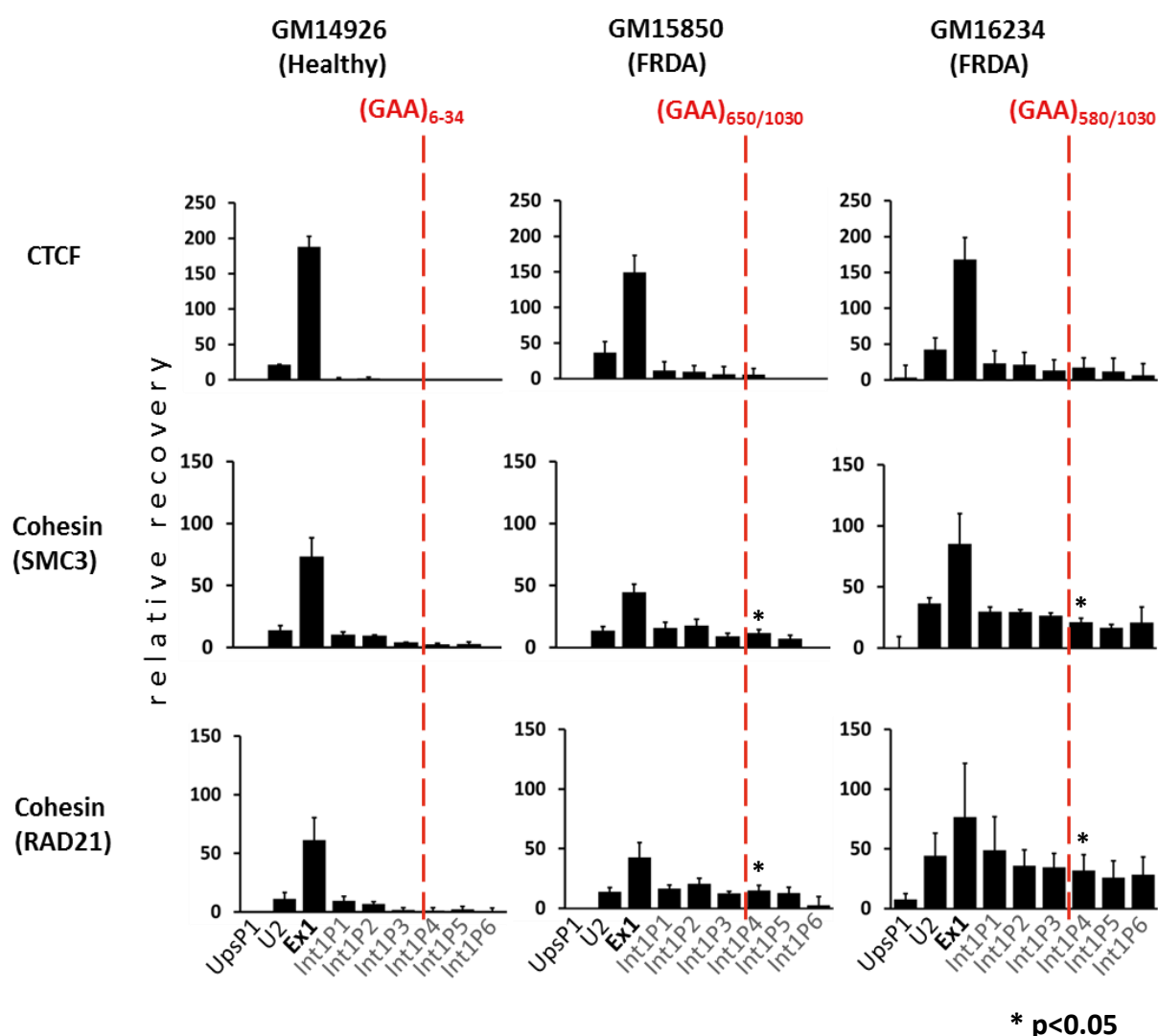


Figure 2.4 – CTCF and cohesin binding at the first part of *FXN* locus. The prototype chromatin insulator CTCF and cohesin ring subunits were found to be bound particularly on Ex1 both in healthy and disease cells. [ChIP signal was shown as recovery percentage relative to H3. * Student's t-test for testing the difference in the ChIP signal from the (GAA)_n flanking Int1P4 region between the healthy and FRDA cells. Error bars: SEM, n=3.]

2.2.3 The sirtuin inhibitor nicotinamide upregulates pathologically-silenced *FXN* by alleviating (GAA)_n induced heterochromatin

Most of the clinical therapeutics for FRDA patients so far have been addressing the symptoms of the disease rather than its actual trigger, which is the abnormal heterochromatin caused by (GAA)_n repeats as shown by others [see introduction 2.1.6] and results presented in figure 2.3. Recent studies revealed the possibility of HDAC inhibitors to relieve the pathological heterochromatin in disease. Although classical broad-range HDAC inhibitors including TSA or SAHA failed to upregulate the silenced *FXN*, Hermen *et al.* (2006) first described a synthetically developed class I HDAC inhibitor BML-210 to be successful in doing so. However, neither BML-210 nor its derivatives reached clinical trial phase yet. Immediately before this Ph.D. study started, preliminary data by Nadine Rothe suggested that the sirtuin inhibitor nicotinamide can also upregulate the pathologically silenced *FXN* in human derived cells at a dose of 10mM (Ph.D. thesis-Rothe, 2008). Importantly, nicotinamide is the amide form of Vitamin B3 and is naturally present in the human body [see general introduction 1.3.2.4]. It has already been used for long periods at high-dose in an attempt to prevent Type – I diabetes in at –risk individuals (Gale *et al.*, 2004). Therefore, it was decided to pursue the potential of nicotinamide as a treatment for FRDA.

In order to test nicotinamide's effect on *FXN* expression, EBV-transformed cell lines were treated for three consecutive days with 10mM nicotinamide. Although there was little effect on the healthy cell line, a moderate level of upregulation (up to ~1.8 fold) was detected in the disease cell lines at the mRNA level by Q-RT-PCR [Figure 2.5A, left panel]. Protein levels were also measured from the same sample by the commercially available *FXN* Mitosciences dipstick assay. In agreement with the mRNA result, *FXN* protein levels were upregulated in the disease cells but not in the healthy cell line [Figure 2.5A right panel; also see supplementary figure S.6A]. The upregulation in FRDA cells at the protein level was up to ~3 fold and brought protein levels to asymptomatic FRDA carrier levels (green line). Interestingly, there was a decrease at the protein level in the healthy cell line, although there was little upregulation at the mRNA level. This will be addressed further in the discussion.

To assess the potential of nicotinamide in a condition closer to normal physiology, resting primary lymphocytes obtained from 4 healthy individuals and FRDA patients were similarly treated with nicotinamide, in cell culture for three consecutive days. In a similar fashion to cell lines, a relatively small effect (~2 fold) was observed for the healthy primary cells at the mRNA level as opposed to a higher (~4.5 fold) response in FRDA primary cells [Figure 2.5B. left panel]. A very small increase (~1.1 fold) was observed for healthy cells at the protein

level, whereas a higher increase (~2.6 fold) was determined in the case of FRDA [Figure 2.5B right panel, also see supplementary figure S.6B].

FRDA is known to affect central and peripheral nerve tissues as well as heart. In order to address this, YG8 transgenic mice which provide a mouse model for FRDA [see introduction 2.1.4 (Al-Mahdawi *et al.*, 2004)] were injected with nicotinamide. Although the LD₅₀ value for nicotinamide was calculated as 2500 mg/kg in mice (Hoffer, 1967), experimental mice in this study showed significant symptoms of side effects at doses higher than 1000 mg/kg. Therefore, a safer dose of 750 mg/kg (~6.14 mM) nicotinamide in physiological saline was introduced to YG8 transgenic mice intraperitoneally once a day for 5 consecutive days, whereas the untreated control group only received the same volume of saline. Analysis of mRNA levels by Q-RT-PCR with human *FXN* transgene specific primers revealed a significant upregulation in nerve tissues including forebrain, spinal cord and cerebellum up to ~1.7 fold [Figure 2.5C left panel]. Protein levels were consistent with the mRNA expression, with the highest upregulation in the spinal cord (~2.1 fold) [Figure 2.5C right panel, also see supplementary figure S.6C]. However, no significant upregulation was found in heart tissue both at mRNA and protein levels.

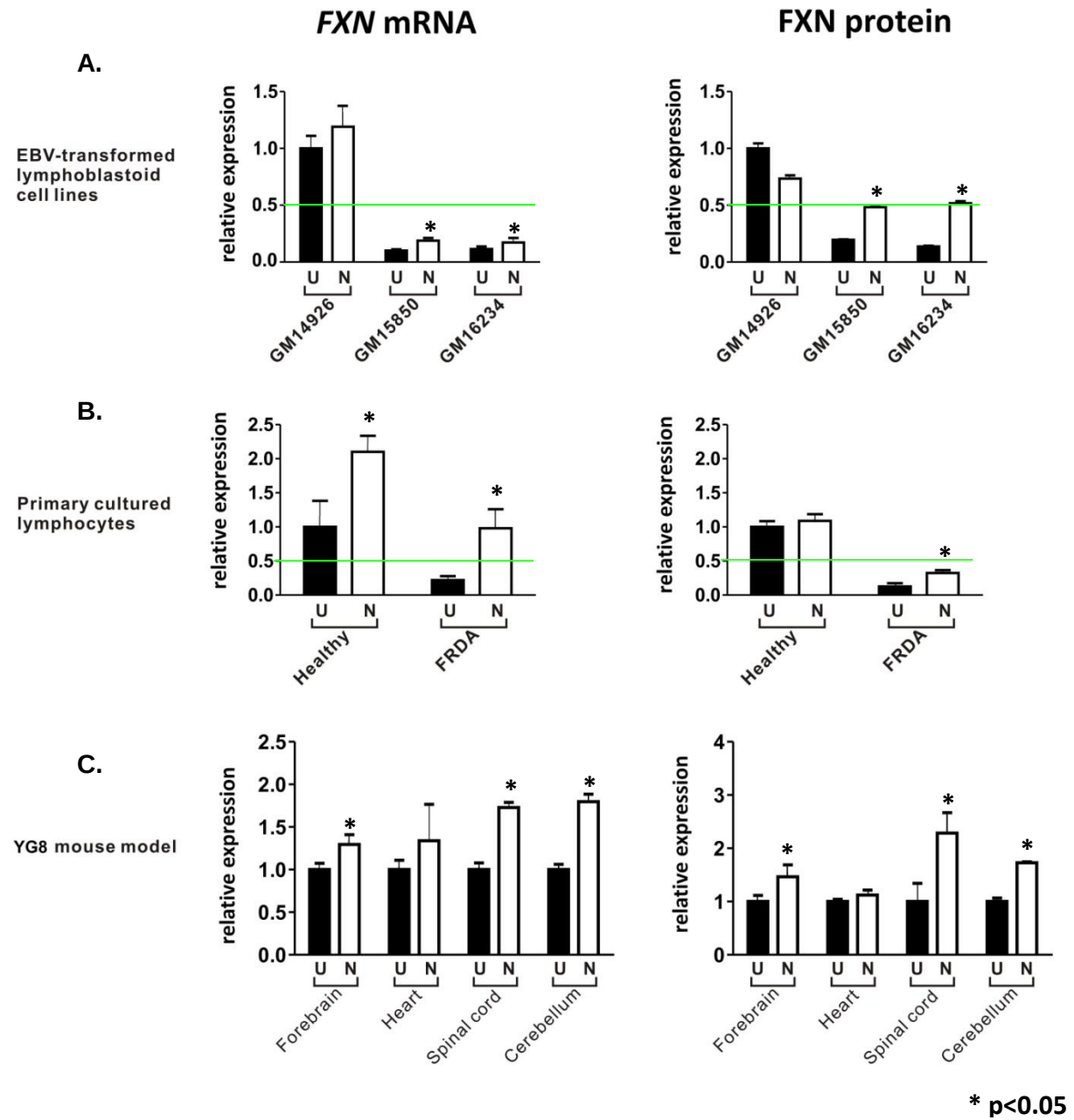
Next, the effect of nicotinamide on chromatin was evaluated by ChIP using antibodies against the heterochromatin marks H3K9me3 and H3K27me3. ChIP was performed on primary lymphocytes after nicotinamide treatment. The enrichment levels of heterochromatin marks in the untreated group were consistent with what was presented in figure 2.3. Higher H3K9me3 levels were noted to be present in Ex1 of primary cells compared to FRDA cell lines. Consistent with the trend observed in figure 2.5, lymphocytes obtained from healthy individuals did not show a marked change in the level of heterochromatin marks whereas FRDA primary cells displayed significantly reduced H3K9me3 and H3K27me3 levels after nicotinamide treatment [Figure 2.6]. Notably, the high level of H3K9me3 in Ex1 almost completely disappeared in FRDA cells following the treatment. In order to see the effect on chromatin *in vivo*, the same cerebellum samples obtained from YG8 mice [as presented in figure 2.5C] underwent ChIP against heterochromatin marks. Consistent with what was observed for primary cells, both H3K9me3 and H3K27me3 levels were substantially reduced following the nicotinamide treatment [Figure 2.7].

The original hypothesis behind the potential of HDAC inhibitors in FRDA therapy is based on the fact that acetylation levels could be increased by preventing histone deacetylation and hence subsequent methylation (review-Festenstein, 2006). To this end, overall histone H3 and H4 acetylations were demonstrated to be increased with nicotinamide treatment on primary lymphocytes obtained from FRDA patients [Figure 2.8]. The patterns of overall

histone acetylations were noted to peak in Ex1 and diminish around the (GAA)_n repeats in contrast to the heterochromatin marks presented in Figure 2.3 and 2.6.

Overall, results in this section showed that nicotinamide relieves the heterochromatinisation of *FXN* gene in the context of pathologically expanded (GAA)_n repeats both *ex vivo* and *in vivo*.

Figure 2.5



See next page for figure legend.

Figure 2.5 – The effect of nicotinamide on FXN expression *in vitro*, *ex vivo* and *in vivo*.

[A] Nicotinamide treatment on cell lines. EBV-transformed lymphoblastoid cells derived from a healthy individual and two FRDA patients as explained in figure 2.3 were treated with nicotinamide (10mM) for 3 consecutive days. Cells were collected upon the treatment and total RNA and protein extraction was performed with the Trizol® protocol. Q-RT-PCR analysis was performed on the cDNA synthesised from the total RNA and FXN protein levels were measured with the Mitosciences dipstick assay. Nicotinamide upregulated *FXN* in FRDA cell lines at both the mRNA and protein level. A small increase was determined upon nicotinamide treatment of the healthy cell line at the mRNA level; surprisingly, this was accompanied by a small decrease in FXN protein levels.

[B] Nicotinamide treatment on primary lymphocytes. Same procedure as described in (A). In agreement with (A), nicotinamide upregulated FRDA in primary cells both at the mRNA and protein level. The extent of upregulation was much lower in the healthy cells compared to cells from patients.

[C] Nicotinamide treatment of YG8 mice. Mice were injected intraperitoneally with 750 mg/kg nicotinamide in physiological saline whereas untreated controls only received saline. This was repeated for 5 consecutive days and tissues were collected on the 6th day; 4 hours after a final 6th injection. Nerve tissues forebrain, cerebellum and spinal cord upregulated *FXN* both at mRNA and protein levels. No such effect was observed for heart. [Left panels show *FXN* mRNA expression levels relative to untreated healthy control after being normalised against β -actin expression. Right panels are FXN protein levels relative to untreated healthy. Green line indicates expression levels in the asymptomatic FRDA carriers. $p < 0.05$ by student's t-test for those indicated with * compared to untreated controls for each. Error bars: SEM, n=3 for (A) and (C) whereas n=4 for (B).]

Figure 2.6

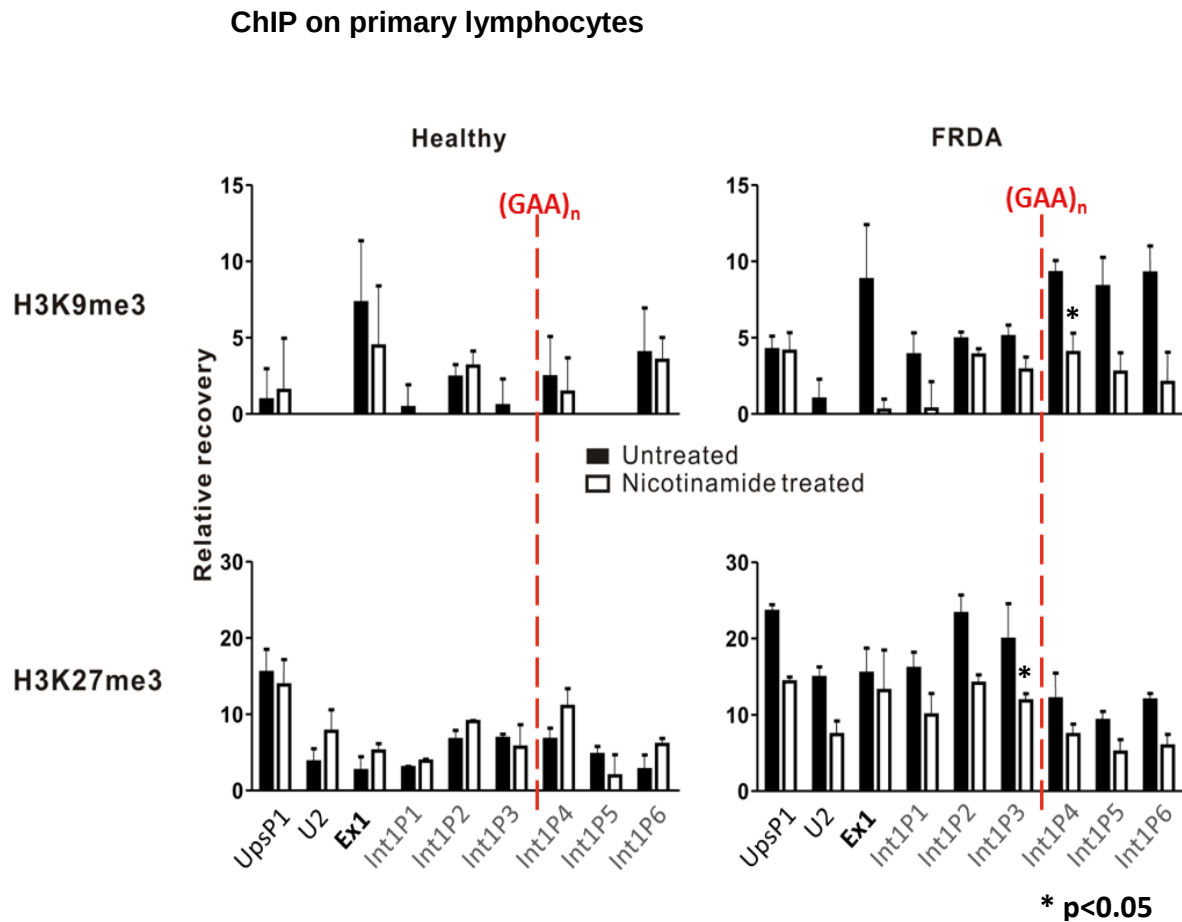


Figure 2.6 – Nicotinamide effect on heterochromatin marks at *FXN* in primary lymphocytes.

The same samples as described in figure 2.5 (from primary lymphocytes obtained from healthy and FRDA individuals) underwent ChIP against heterochromatin marks H3K9me3 and H3K27me3. A marked decrease was observed for both heterochromatin marks in the FRDA cells whereas no significant effect was observed on healthy cells. [ChIP signal was shown as recovery percentage relative to H3. p<0.05 by student's t-test for those indicated with * in comparison to the untreated level for the specific primer set. Error bars: SEM, n=4]

Figure 2.7

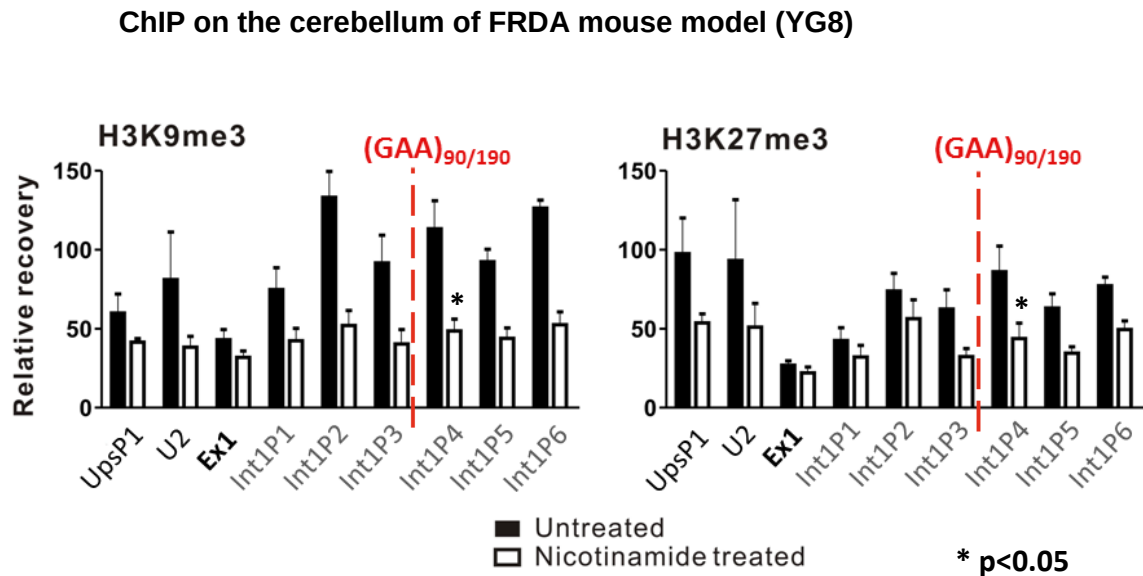


Figure 2.7 – Nicotinamide effect on heterochromatin marks at *FXN* in YG8 mouse tissues.

The same cerebellum samples (as described in figure 2.5) underwent ChIP against heterochromatin marks H3K9me3 and H3K27me3. A marked decrease was observed for both heterochromatin marks in the FRDA mouse model cerebellum in agreement with the increase in *FXN* expression levels presented in figure 2.5 [ChIP signal was shown as recovery percentage relative to H3. $p < 0.05$ by student's t-test for those indicated with * in comparison to the untreated level for the specific primer set. Error bars: SEM, $n=3$.]

Figure 2.8

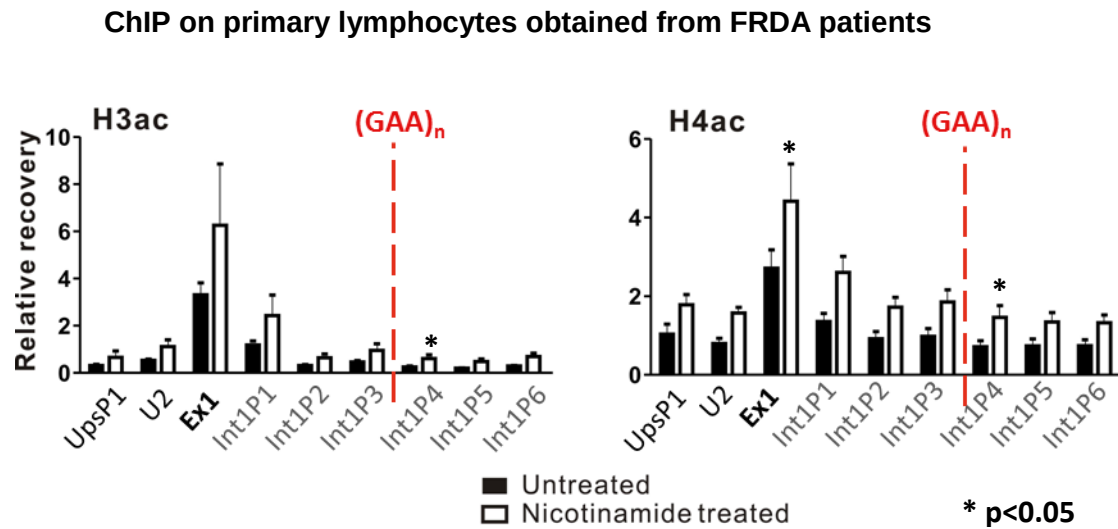


Figure 2.8 – Nicotinamide effect on chromatin acetylation at *FXN* in primary lymphocytes.

The same samples of primary lymphocytes presented in figure 2.5 and 2.6 underwent ChIP against overall histone H3 and H4 acetylations. Acetylation levels appear inversely correlated with the previously-presented heterochromatin marks, peaking in Ex1. A marked increase was observed for both total acetylation marks upon nicotinamide treatment in the FRDA primary lymphocytes particularly in Ex1, in agreement with the increase in *FXN* expression levels presented in figure 2.5 [ChIP signal was shown as recovery percentage relative to H3. $p < 0.05$ by student's t-test for those indicated with * in comparison to the untreated level for the specific primer set. Error bars: SEM, $n=4$.]

2.2.4 RNA polymerase II is stalled within the first exon of *FXN*

What is the exact effect of heterochromatin on the dysregulation of *FXN* in disease? Heterochromatin is classically thought to silence genes by restricting the access of RNAPII and transcription factors to the genetic code [see general introduction 1.2.2]. In the case of FRDA, results in this chapter so far provided evidence that heterochromatin is present in close proximity to the expanded (GAA)_n repeats; however, the UTR region as well as the first exon seemed to be considerably less invaded by heterochromatin. Based on this, one may expect that the recruitment of RNAPII to the promoter may not be the primary restricting step for the production of *FXN* transcripts. Indeed, ChIP results with a chromatin mark that correlates with active transcription H3K4me2 [see general introduction 1.3.3.2] revealed no difference between the healthy and disease cell lines within the Ex1, where it peaks [Figure 2.9 upper panel]. Overall, it seemed more likely that silencing may result at least in part from a defect in transcription elongation as the RNAPII comes across the heterochromatin barrier. Consistent with this possibility, Punga *et al.* (2010) suggested a transcription elongation problem using mRNA turn-over assays and H3K36me3 ChIP. However, the position of this defect was not studied and was assumed to occur within the GAA-repeat expansion itself.

In this project, specific and extensively tested antibodies [personal communication with Ana Pombo and Emily Brookes, MRC CSC -London] were used in order to determine the distinct distribution patterns for the initiating and elongating forms of RNAPII. As explained in the general introduction 1.6.3 and 1.6.4 [see figures 1.17 and 1.18], the initiating form of RNAPII is phosphorylated at the serine-5 (S5p) residue of its carboxy-terminal domain (CTD) and is typically enriched at the promoter regions of active and paused genes. On the other hand, the elongating form of RNAPII is phosphorylated at the serine 2 (S2p) CTD residue and is known to be enriched highly in the gene bodies of active genes. ChIP experiments with S5p and S2p specific RNAPII antibodies revealed an interesting distribution across the *FXN* gene both in healthy and FRDA cell lines [Figure 2.9 middle and lower panels]. The S5p form peaked within the Ex1 and the signal was no lower in the disease cell lines. Intriguingly, S2p form also peaked at the same place which is inconsistent with its typical distribution pattern at active genes where it is enriched steadily through the coding regions [General introduction figure 1.18]. This observation was also confirmed in *ex vivo* primary cells as shown in supplementary figure S.7. Such an unusual distribution of RNAPII at the *FXN* gene could be explained RNAPII stalling within the first exon both in healthy and disease conditions, in agreement with a recent study by Kim *et al.* (2011) which reported more primary transcript upstream than downstream of this newly identified site within the Ex1 where RNAPII has accumulated. Interestingly, S2P RNAPII signal from the Ex1 primer was higher in FRDA cell lines compared to the healthy cell line. However, this was not observed in the primary cells.

To pursue this further, RNA immunoprecipitation (RIP) was performed. Here, RNAPII is immunoprecipitated in the same way as in ChIP; however, RNA is recovered instead of DNA. This allows one to study on-going transcription and gives a better insight in terms of the progress of transcription along a certain region. S2p RNAPII precipitated and recovered RNA was used to synthesise cDNA using random hexamers. Next the signal was quantified using primers with same efficiency rates [Figure 2.10 upper panel]. Notably, a ~6 fold enrichment was determined for RNA transcribed from Ex1 compared with a region ~600bp downstream within the first intron [Figure 2.10 lower panel]. Although this RIP experiment suggested that RNAPII S2p could indeed be stalling in Ex1, a more detailed approach was needed in order to strengthen this hypothesis.

In order to address the stability and dynamics of RNAPII more directly, advantage was taken from two known inhibitors of RNAPII. Alpha-amanitin is a strong inhibitor of RNAPII. It interacts with the bridge helix in the core enzyme and interfering with its translocation (Chafin *et al.*, 1995; Gong *et al.*, 2004; Lee *et al.*, 2002; Nguyen *et al.*, 1996; Rudd and Luse, 1996; Xie *et al.*, 2006). On the other hand, 6-dichloro-1 β -ribofuranosylbenzimidazol (DRB) inhibits the kinase activity of CDK9 associated with the positive elongation factor b (P-TEFb) on the negative elongation factor NELF and the S2 residue of RNAPII (Chodosh *et al.*, 1989; Dubois *et al.*, 1994; Marshall *et al.*, 1996; Xie *et al.*, 2006). Both of these classic RNAPII inhibitors disrupt the progress of transcription into the coding regions by stopping the incoming RNAPII complexes from the promoter. This allows the estimation of elongation rates of RNAPII at certain regions via ChIP. Typically, successfully elongating RNAPII complexes show sensitivity to these inhibitors and stalled RNAPII complexes with low turnover rates do not exhibit any sensitivity (Gong *et al.*, 2004; Stock *et al.*, 2007; Xie *et al.*, 2006). Here, healthy and disease cell lines were treated with 75 μ g/ml alpha-amanitin and 50 μ M DRB for 7 hours based on previous references mentioned above and personal communication with Julie Stock and Ana Pombo (MRC CSC-London). On-going transcription was inhibited effectively as primary *FXN* transcripts (Int3-Ex4) were diminished significantly after the treatments in all cell lines [Figure 2.11A]. To test the dynamics of RNAPII, the abundance of both S5p and S2p forms of RNAPII were studied by ChIP on the *FXN* locus after successful transcriptional inhibition. For the healthy cell line, treatment with both drugs induced a slight increase in RNAPII-S5p enrichment at Ex1, whereas the levels of RNAPII-S2p were decreased only slightly [Figure 2.11B]. As DRB directly inhibits the S2 phosphorylation, the unaffected RNAPII-S2p peak in the healthy cell line should represent a stable pool of RNAPII complexes that are not undergoing cycles of S2 phosphorylation. This suggests that the accumulation of RNAPII on Ex1 is not due to a minor reduction in transcriptional elongation across this region, but instead most of these complexes remain

stalled in the healthy cell line throughout the 7 hours treatment time. Moreover, the resistance of RNAPII-S2p peak to alpha-amanitin treatment implies that this stalling effect in the healthy cell line is unusual as persistently stalled RNAPII-S2p is typically a target for proteasomal degradation. It was also shown that elongating RNAPII-S2p stalls when it encounters DNA damage and is then removed by the proteasome; a phenomenon shown in transcriptional-coupled DNA repair (Anindya *et al.*, 2007; Bregman *et al.*, 1996; Lee *et al.*, 2002; Mitsui and Sharp, 1999; Somesh *et al.*, 2005; Somesh *et al.*, 2007; Woudstra *et al.*, 2002). Therefore, RNAPII is not only stalled in the Ex1 of *FXN* in the healthy cells but also unusually refractory to S2p-dependent degradation by the proteasome. Given the fact that the gene is normally transcribing at low levels (Campuzano *et al.*, 1997; Su *et al.*, 2004), one may deduce that there should be an intermittent or continuous release of RNAPII from this stall site, making it a proximal point at which the progress of RNAPII is regulated.

In contrast to healthy cell line, RNAPII-S2p levels at Ex1 showed sensitivity to both transcriptional inhibitors in disease cell lines; to a higher extent for GM16234 which expresses *FXN* at even lower levels (see figure 2.2B). Moreover, RNAPII-S5p levels did not significantly respond to any treatment suggesting that most stalled RNAPII molecules identified at the *FXN* locus are enriched in S5p but not in S2p. Because the disease cell lines express much less *FXN* compared to healthy, the reduction in RNAPII-S2p following transcriptional inhibition with DRB could not easily be explained by increased rates of elongation in patients (the RNAPII that is already phosphorylated on S2P prior to DRB treatment remains elongation competent – so in principle its disappearance from this site could be due to elongation). Importantly, the RNAPII-S2p peak was also reduced in the presence of alpha-amanitin (which blocks all movement of RNAPII). This implied that the removal of RNAPII-S2P was taking place by a pathological mechanism in the FRDA cells as it did not take place in normal cells. This was addressed further in the following section.

Figure 2.9

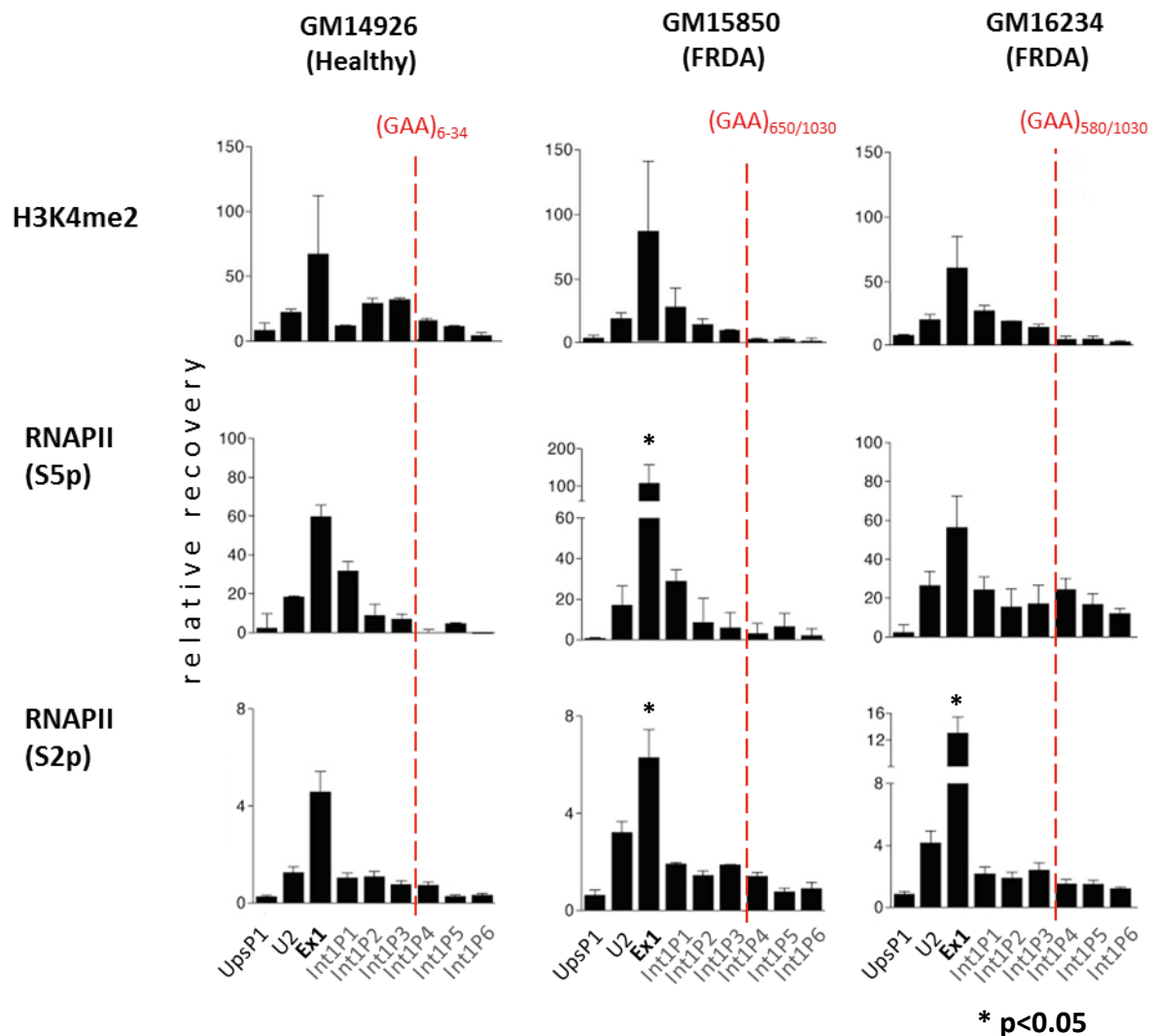


Figure 2.9 – H3K4me2 and RNAPII levels at the first part of *FXN* locus. ChIP was performed on the healthy and FRDA cell lines using highly specific antibodies against the newly transcribed chromatin mark H3K4me2 and initiating (S5p) and elongating (S2p) forms of RNAPII. Both H3K4me2 and RNAPII levels peaked in Ex1 of all cell lines and this was not lower in the disease cell lines compared to healthy. The experiment was repeated in *ex vivo* primary cells as presented in supplementary figure S.7. [ChIP signal was shown as recovery percentage relative to H3. * Student's t-test in comparison to Ex1 recovery signal from the healthy cell line. Error bars: SEM, n=3.]

Figure 2.10

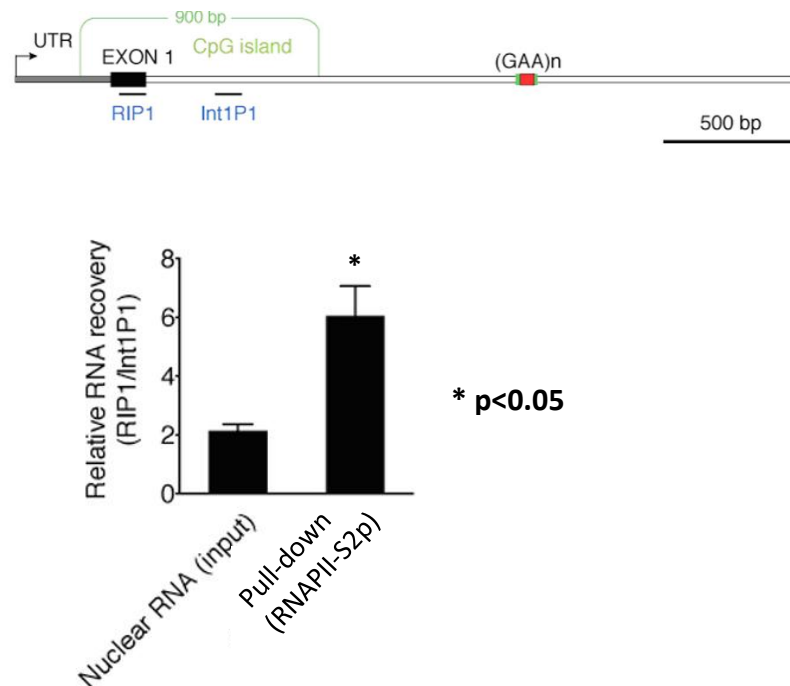


Figure 2.10 – RNA immunoprecipitation (RIP) on *FXN*. RIP was performed using the same RNAPII-S2p antibody mentioned in figure 2.9. cDNA was synthesised from the RNA extracted from the immunoprecipitated material using random hexamers. Primary transcript levels were measured by Q-RT-PCR using primers indicated in the upper panel. Crucially both of these primers have the same efficiency rates. Nuclear and immunoprecipitated RNA was analysed and the signal ratio (RIP1/Int1P1) was revealed to be >1 in both groups suggesting transcriptional stalling in Ex1. Notably, there was a significantly greater enrichment in the RIP1 signal compared to Int1P1 signal for the immunoprecipitated material. [* Student's t-test in comparison to the nuclear input. Error bars: SEM, n=4]

Figure 2.11

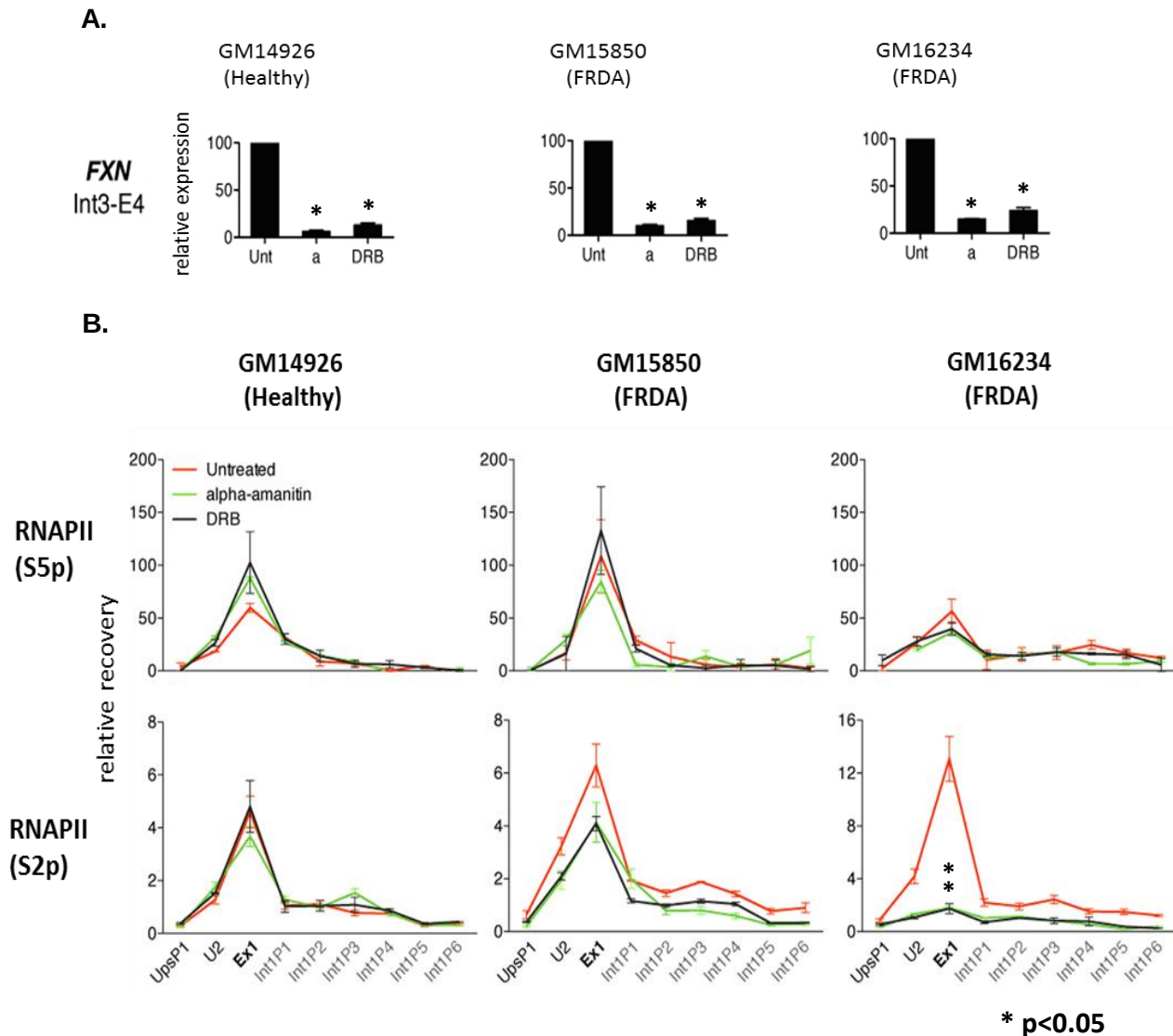


Figure 2.11 – Transcriptional inhibition by alpha-amanitin and DRB. Healthy and FRDA cell lines were treated 7 hours in culture with classic transcription inhibitors alpha-amanitin (75 $\mu\text{g/ml}$) and DRB (50 μM). Alpha-amanitin blocks the translocation of elongating RNAPII whereas DRB inhibits the S2p phosphorylation needed for an efficient elongation complex. **[A] Transcript levels post treatment.** As expected, treatments significantly diminished *FXN* primary transcripts indicating that the transcription was indeed inhibited. Exactly the same amount of RNA was used – levels are expressed relative to untreated. [* Student's t-test in comparison to the untreated sample. Error bars: SEM, n=3.] **[B] RNAPII ChIP post treatment.** While there was no significant sensitivity at RNAPII-S5p levels (upper panel), both disease cell lines exhibited decreased RNAPII-S2p levels upon transcriptional inhibition. However, the RNAPII-S2p levels in the healthy cell line were insensitive to any treatment implying a persistent stalling that is refractory to proteasome-dependent degradation. [ChIP data presented as percentage to H3. *Student's t-test in comparison to the recovery rate from the untreated sample. Error bars: SEM, n=3]

2.2.5 Stalled RNA polymerase II gets degraded by the proteasome at the pathologically silenced *FXN*

Considering the low levels of *FXN* transcription in FRDA, the sensitivity of RNAPII-S2p to transcriptional inhibition in disease cell lines but not in healthy might be explained by the proteasomal degradation of stalled RNAPII-S2p complexes in FRDA as had previously been seen with stalled RNAPII-S2P in transcription-coupled DNA repair (Anindya *et al.*, 2007; Bregman *et al.*, 1996; Lee *et al.*, 2002; Mitsui and Sharp, 1999; Somesh *et al.*, 2005; Somesh *et al.*, 2007; Woudstra *et al.*, 2002). This possibility was investigated using ChIP against the 19S proteasome which revealed an enrichment of proteasome binding within the *FXN* locus in the disease cell lines compared to healthy [Figure 2.12A]. The binding of proteasome was also investigated with 20S catalytic particle specific antibodies; however, no such antibody was identified to be efficient for the ChIP technique [see supplementary table S.2]. There is an interesting pattern for the binding of the 19S regulatory particle, whose presence is needed for substrate recognition and successful proteasomal degradation of large proteins such as RNAPII [see general introduction 1.7.1]. Notably, proteasome ChIP signal peaked moderately at Ex1 and in a region immediately downstream of (GAA)_n repeats in all cell lines. Interestingly there was also a more pronounced peak in the case of expanded (GAA)_n repeats, in a similar pattern to that obtained for the heterochromatin components H3K9me3 and HP1 presented in figure 2.3. This finding was also confirmed on *ex vivo* primary cells [see supplementary figure S.8].

Proteasome ChIP suggested that (GAA)_n repeats could somehow recruit proteasome to the *FXN* locus, providing an important step in FRDA pathology by rendering the stalled RNAPII-S2p more susceptible to degradation. To test this hypothesis, the effect of proteasome inhibition was measured on RNAPII levels upon transcriptional inhibition by alpha-amanitin, which stops incoming RNAPII complexes to Ex1 and allows one to study the stability of RNAPII within this region. For this purpose, well-known proteasome inhibitors MG132 and PS341 were employed [see general introduction 1.7.2]. Importantly PS341 is already in clinical use to treat patients with multiple myeloma. Initially, the culture medium of healthy and FRDA cell lines were added with the proteasome inhibitor PS341 at a concentration of 10µM and alpha-amanitin (75 µg/ml) was supplemented one hour later. This was done in order to give some time for the proteasomal inhibition to take place before the transcriptional inhibition. After the addition of alpha-amanitin, cells were treated for an additional 7 hours in the presence of both drugs. RNAPII-S2p levels were then measured with ChIP. Notably, PS341 prevented the alpha-amanitin induced depletion of RNAPII-S2p in Ex1 in both FRDA cell lines most markedly in GM16234 [Figure 2.12B]. This was consistent with the hypothesis that the dysregulation of *FXN* in FRDA was proteasome-dependent. In contrast, no PS341

effect was observed on the healthy cell line which independently confirmed the refractory nature of RNAPII-S2p to proteasomal degradation in normal individuals.

Because RNAPII-S2p levels could be recovered at Ex1 with the PS341 treatment, it was envisaged that inhibiting the proteasome may also help to upregulate *FXN* expression in FRDA by restoring the reservoir of RNAPII available for transcription. In order to address this, cell lines were similarly treated with 10 μ M PS341 for 8 hours and *FXN* transcript levels were measured by Q-RT-PCR. There was a dramatic increase in *FXN* primary transcripts (Int3-Ex4) upon PS341 treatment in the disease cell lines, again to a higher extent in GM16234 [Figure 2.12C left panel]. This was bringing the primary transcript level in the FRDA cells up to the levels of healthy cell line. Similarly, *FXN* mRNA levels also increased up to the level of asymptomatic heterozygous FRDA carriers [Figure 2.12C right panel]. On the other hand, no significant effect was observed for the healthy cell line both at pre-mRNA and mRNA levels, consistent with the ChIP results. Responses at the protein level were in broad agreement with the mRNA result [see supplementary figure S.9]. Together; these results confirmed that susceptibility of stalled RNAPII to proteasomal degradation is a pathological phenomenon involved in the dysregulation of the *FXN* gene in the presence of expanded (GAA)_n repeats.

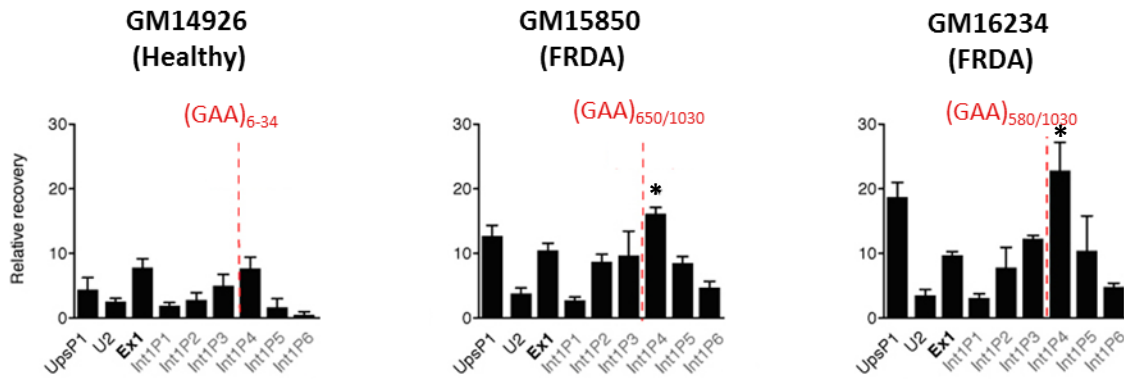
ChIP results were also reproduced using another laboratory-standard proteasome inhibitor, MG132 [Figure 2.13A]. This time a new healthy cell line GM15851 was also included to the experimental set. Consistent with PS341 effect, both healthy cell lines were refractory to proteasome-dependent degradation of stalled RNAPII-S2p complexes within Ex1. In contrast, RNAPII-S2p levels in the FRDA cells were sensitive to proteasomal degradation and this could be restored by the proteasome inhibitor MG132. Again, proteasome inhibition upregulated both primary *FXN* transcripts and *FXN* mRNA significantly [Figure 2.13B]. In order to see if the extent of upregulation was consistent with the recovery rates of RNAPII-S2p upon proteasome inhibition, a statistical analysis was performed using MS Excel software [Figure 2.13C]. Here, a recovery index was calculated by dividing the Ex1 ChIP signal obtained by alpha-amanitin+MG132 treatment with the signal obtained by alpha-amanitin only. Box plot analysis and Mann-Whitney U test revealed that the effect of proteasome inhibition is highly specific to FRDA cells but not healthy ($p < 0.05$) [left panel]. The recovery index was also correlated with the fold change of upregulation on a scatter plot by linear regression analysis [right panel]. A significantly positive correlation was determined by the regression analysis ($R^2 = 0.976$).

Overall, results here showed that the elongating form of RNAPII (S2p) is stalled within the Ex1 in normal conditions. This stalling seems to be unusually refractory to proteasome-dependent degradation. However, it becomes susceptible to proteasomal degradation in the presence of expanded (GAA)_n repeats, possibly because of the elevated levels of proteasome binding. This could be restored by proteasome inhibitors, which opens a potential new avenue of therapeutics for the incurable Friedreich's ataxia.

Figure 2.12

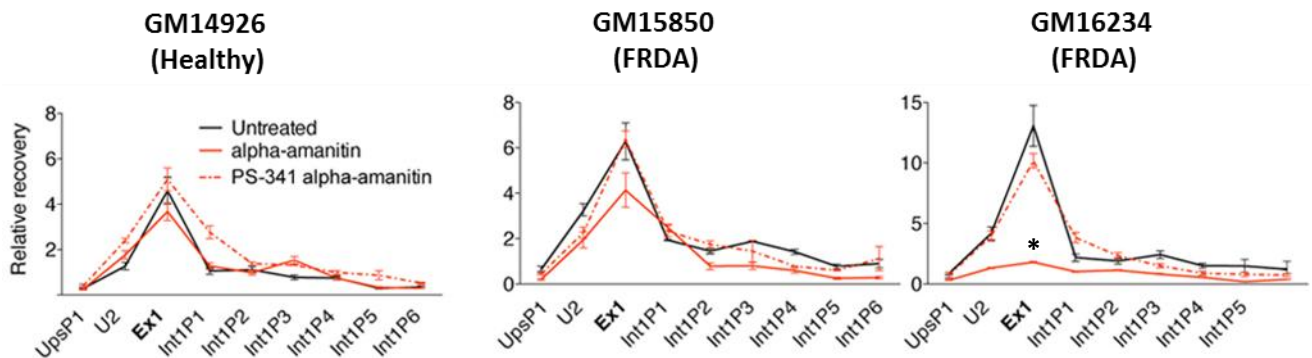
A.

19S proteasome ChIP

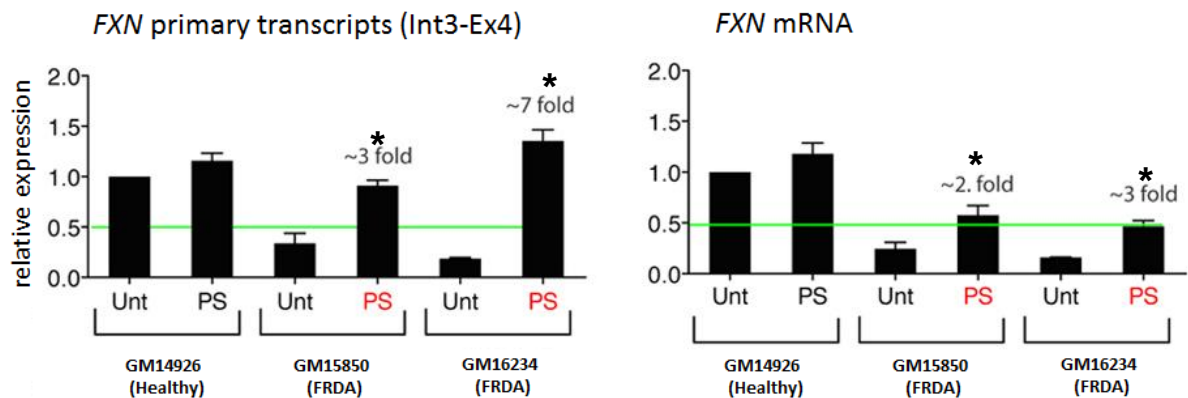


B.

RNAPII-S2p ChIP



C.



* $p < 0.05$

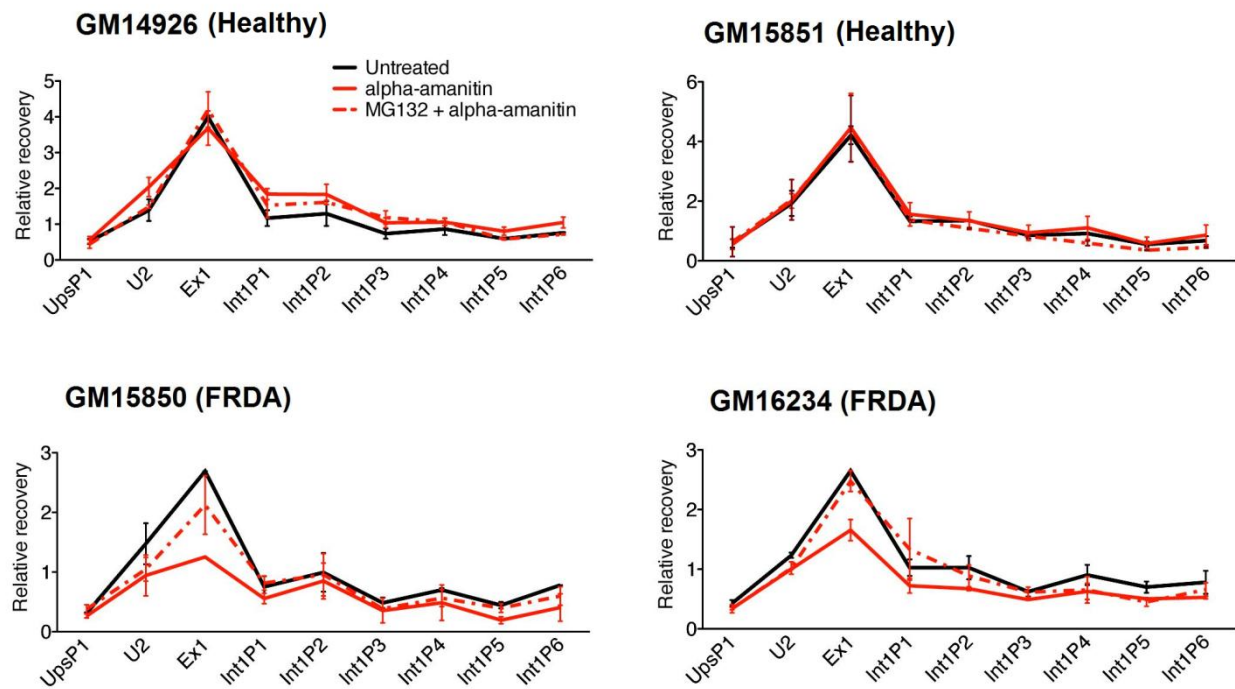
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Figure 2.12 – ChIP and expression analyses to study the effect of proteasome inhibition by PS341 on the stalled RNAPII. [A] **Proteasome ChIP.** First part of *FXN* locus was investigated in terms of proteasome binding using ChIP against the 19S proteasomal subunit RPN10. Binding was significantly enriched in the disease cell lines compared to healthy. Interestingly, proteasome signal resembles H3K9me3 and the HP1 binding patterns presented in figure 2.3. [ChIP signal was shown as recovery percentage relative to H3. $p < 0.05$ by student's t-test for those indicated with * in comparison to the signal from the same primer in the healthy cell line. Error bars: SEM, $n=3$] [B] **RNAPII-S2p ChIP.** Here, cells were treated with the transcription inhibitor alpha-amanitin both in the presence and absence of proteasome inhibitor PS341 (1 hour with 10 μ M PS341 only and then 7 hours with 75 μ g/ml alpha-amanitin + 10 μ M PS341). Proteasome inhibition could restore the RNAPII-S2p levels in the FRDA cells upon transcriptional inhibition, whereas no effect was observed in the healthy cell line. [* Student's t-test in comparison to the recovery rate from the untreated sample. ChIP data presented as percentage to H3. Error bars: SEM, $n=3$] [C] ***FXN* expression upon proteasome inhibition.** PS341 treatment upregulated both primary *FXN* transcript and mRNA significantly to potentially therapeutic levels. [Data was presented as relative expression to the value obtained from untreated healthy cells. Housekeeping normalisation was done against β -actin. Green line indicates the relative expression levels of asymptomatic heterozygous FRDA carriers. $*p < 0.05$ student's t-test in comparison to the expression levels from the untreated samples. Error bars: SEM, $n=3$.]

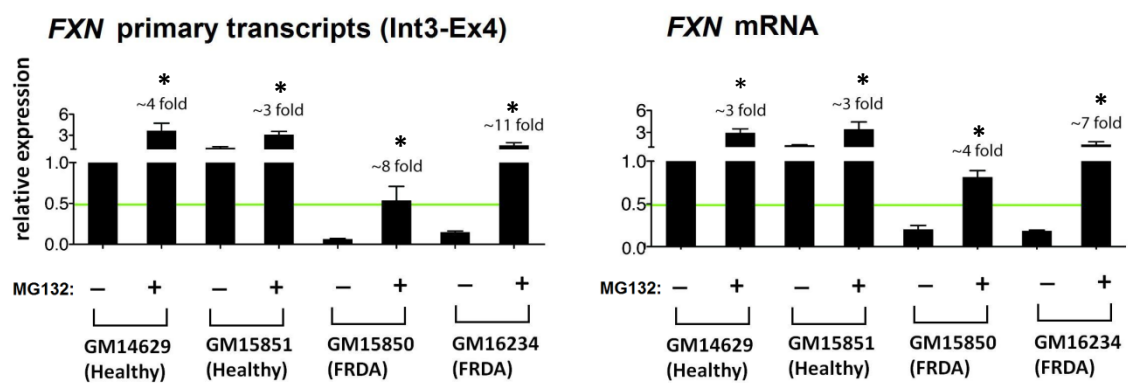
Figure 2.13

RNAPII - S2p ChIP

A.

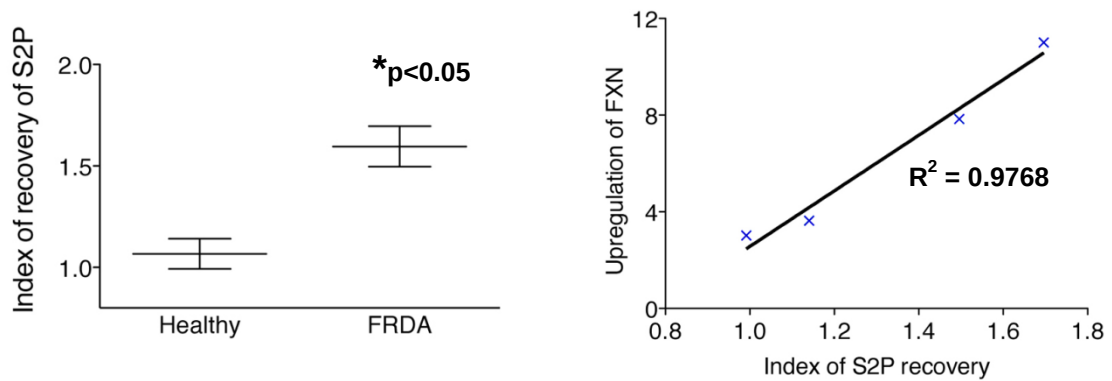


B.



* $p < 0.05$

C.



See next page for figure legend.

Figure 2.13 – ChIP and expression analyses to study the effect of proteasome inhibition by MG132 on the stalled RNAPII. [A] **RNAPII-S2p ChIP.** Here, cells were treated with the transcription inhibitor alpha-amanitin both in the presence and absence of proteasome inhibitor MG132 (1 hour with 10 μ M PS341 only and then 7 hours with 75 μ g/ml alpha-amanitin + 10 μ M MG132). ChIP experiments showed that proteasome inhibition could restore the RNAPII-S2p levels in the FRDA cells upon transcriptional inhibition, whereas no effect was observed in the healthy cell line. [Error bars: SEM, n=3, ChIP data presented as percentage to H3.] [B] ***FXN* expression upon proteasome inhibition.** MG132 treatment upregulated both primary *FXN* transcripts and mRNA significantly to potentially therapeutic levels. [Data was presented as relative expression to the value obtained from untreated healthy cells. Housekeeping normalisation was done against *β -actin*. *p<0.05 student's t-test in comparison to the expression levels from the untreated samples. Error bars: SEM, n=3.] [C] **Statistical analysis of MG132 effect.** Here, a recovery index was calculated by dividing the Ex1 ChIP signal obtained by alpha-amanitin+MG132 treatment with the signal obtained by alpha-amanitin only. Box plot analysis and Mann-Whitney U test revealed that the effect of proteasome inhibition is highly specific to FRDA cells but not healthy (p<0.05) [left panel]. The recovery index was also correlated with the fold change of upregulation on a scatter plot by linear regression analysis [right panel]. A strong positive correlation was determined by the regression analysis ($R^2=0.976$).

2.2.6 HP1 γ as a potential regulator of the newly identified RNA polymerase II stalling site on *FXN*

According to the results explained up to this point, the newly identified RNAPII site within the first exon of *FXN* seems to be a crucial point in terms of the gene dysregulation in FRDA. One obvious question to ask was: how is this key site regulated? In order to investigate this further, the scientific literature was reviewed for studies where a transcriptional pausing or stalling effect was observed. It was realised that HP1 γ was found in the promoter-proximal region of the *c-MYC* gene, where transcriptional pausing takes place (Eick *et al.*, 1987; Ogawa *et al.*, 2002; Spilianakis *et al.*, 2003). Moreover, a persistent transcriptional stalling was described previously for the latent phase HIV-LTR (Kao *et al.*, 1987). Interestingly, this site is known to be occupied by HP1 γ which was shown to be crucial for the silencing effect to take place during the virus' latent phase (du Chene *et al.*, 2007). More importantly, this site was also shown to be regulated by the proteasome, although whether it degrades the paused RNAPII was not studied directly (Lassot *et al.*, 2007). In agreement with these observations, Figure 2.3 presented enriched levels of HP1 γ binding in Ex1 of *FXN*, where the stalling of RNAPII takes place. This suggested the possibility that HP1 γ may play a regulatory role at this important site on the *FXN* gene. In order to address this, the FRDA mouse model YG8 (C57BL/6J) was crossed with the heterozygous HP1 γ (C57BL/6J) deficient mice (Fleming *et al.*, 2005b). HP1 γ binding was analysed using ChIP on *ex vivo* cerebellum, for a tissue which can be affected by FRDA. Consistent with the cell lines, a peak of HP1 γ was detected within the Ex1 in cerebellum [Figure 2.14A]. The RNAPII stalling site was also shown to be enriched for RNAPII-S5p in Ex1 [Figure 2.14B]. Unfortunately, RNAPII-S2p ChIP did not work in mouse using the same antibody used with human cells. Notably, HP1 γ deficient (-/+) h*FXN* transgenic mice had more *FXN* mRNA in cerebellum compared to the HP1 γ wild type (+/+) h*FXN* transgenic mice [Figure 2.14C].

Although the mechanism is not yet clear, these results together suggest that HP1 γ may indeed play an important role in the regulation of the *FXN* gene. Possible explanations for this will be made in discussion 2.3.1 and 2.3.3.

Figure 2.14

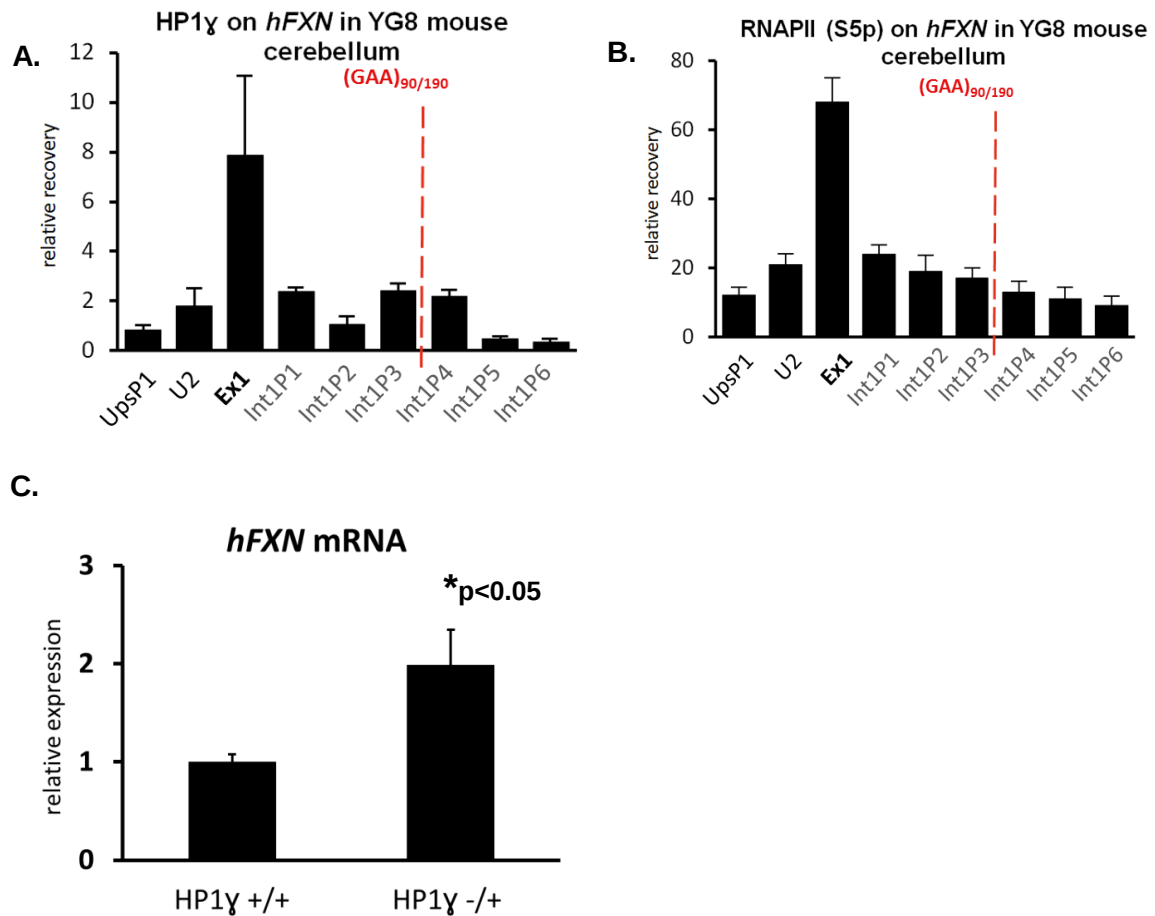


Figure 2.14 – ChIP and *FXN* mRNA expression analysis on HP1γ deficient mice. FRDA mouse model YG8 (C57BL/6J) was crossed with the heterozygous HP1γ (C57BL/6J) deficient mice. Experiments were performed on the cerebellum collected from 4-6 week old mice. **[A] HP1γ ChIP.** The binding of HP1γ is enriched within the Ex1. **[B] RNAPII-S5p ChIP.** Consistently with the cell lines, the signal peaks in Ex1 suggesting that the accumulation effect also takes place *in vivo*. **[C] mRNA expression analysis.** RNA extracted from the same samples with ChIP were synthesised to cDNA and this was quantified by Q-RT-PCR using a primer set that only amplifies human *FXN* transcripts. Comparison with the wild type HP1γ (+/+) *hFXN* transgenic mice showed that the HP1γ (-/+) depleted mice had significantly higher *FXN* mRNA levels. [ChIP results were shown as percentage to H3 signal. Q-RT-PCR result as relative expression to wild type HP1γ *hFXN* transgenic mice after normalisation against β -actin expression. *Student's t-test. Error bars: SEM, n=3.]

2.3 Discussion

2.3.1 Heterochromatinisation of the *FXN* gene in Friedreich's ataxia

As mentioned before, there was only limited evidence regarding the presence of heterochromatin on the endogenous *FXN* locus at the time this Ph.D. started. Here, *in vitro* and *ex vivo* ChIPs with human cells as well as *in vivo* experiments with the YG8 FRDA mouse model clearly demonstrated that expanded (GAA)_n repeats indeed form a heterochromatic centre from which heterochromatin spreads within the *FXN* locus in either direction. This is consistent with other published reports (Al-Mahdawi *et al.*, 2008; De Biase *et al.*, 2009; Greene *et al.*, 2007; Herman *et al.*, 2006; Kim *et al.*, 2011a; Punga and Buhler, 2010).

Two robust heterochromatin marks H3K9me3 and H3K27me3 were shown to be particularly enriched in close proximity to the expanded (GAA)_n repeats [Figure 2.3]. This is perhaps surprising as these two heterochromatic marks do not usually overlap in other genomic regions as shown by Barski *et al.* (2007) and Wang *et al.* (2008). Typically, H3K9me3 is associated with the constitutive heterochromatin and H3K27me3 is linked to the silencing of formerly euchromatic genes via the action of polycomb proteins to form so-called 'facultative' heterochromatin [see general introduction 1.3.3.3 and 1.3.3.4]. Notably, it is known that H3K27me3 may substitute for H3K9me3 in the absence of the H3K9 specific methyltransferase SUV39H (Peters *et al.*, 2003). Whether there is co-operation or redundancy between these two marks at the *FXN* locus is a critical question that remains to be answered. However, the fact that SIRT1 [which deacetylates and activates H3K9 specific HKMT SUV39H (Vaquero *et al.*, 2007)] interacts with the EED component of the PRC2 complex (Kuzmichev *et al.*, 2005) may provide the mechanistic link between the two well-known heterochromatin marks. The enrichment patterns of heterochromatin marks correspond well with the recruitment levels of HP1β [Figure 2.3], which is well known to induce a compact chromatin structure [see general introduction 1.3.3.4]. Interestingly, there was a pronounced binding of HP1β even in the healthy cells adjacent to unexpanded (GAA)_n repeats. However, this could be observed to a much greater extent in the FRDA cells. Moreover, DNase-I hypersensitivity assays performed in Richard Festenstein's laboratory also suggested a highly compact chromatin structure spreading from the (GAA)_n repeats [by Jackson P.K. Chan, MRC CSC-London, data not shown]. According to this data, inaccessibility increases towards the (GAA)_n repeats both in healthy and FRDA cells, taking place to a much greater extent in FRDA. Taken together, it could be suggested that a low level of *FXN* heterochromatinisation is present even in the healthy cells, consistent with the

low physiological expression levels of *FXN* under normal conditions (Campuzano *et al.*, 1997; Su *et al.*, 2004).

In contrast to other heterochromatic factors, the γ isoform of HP1 was not shown to bind to the *FXN* locus at higher levels in the FRDA cells compared to healthy [Figure 2.3], although a former study reported enriched HP1 γ binding levels in the 5' UTR of FRDA cells (De Biase *et al.*, 2009). Notably both the antibody and primer sets used in this projects were different in comparison to the formerly published study. Here, HP1 γ levels peaked within the first exon (Ex1) of the *FXN* locus where the heterochromatin marks seem to be lowered both in healthy and FRDA cells as well as YG8 mouse cerebellum. This suggests that HP1 γ is perhaps needed for the native physiological regulation of *FXN*, consistent with its recently described roles in stabilising nascent transcripts as well as regulating RNAPII traffic in transcriptionally active genes (Kwon *et al.*, 2010; Minc *et al.*, 1999; Minc *et al.*, 2000; Vakoc *et al.*, 2005). In addition, it is still possible that HP1 γ may indirectly contribute to *FXN* silencing by forming heterodimers with other heterochromatic factors such as HP1 β , which was found to be at higher levels in the case of expanded (GAA) $_n$ repeats (Brasher *et al.*, 2000; Cowieson *et al.*, 2000; review-Hiragami and Festenstein, 2005). Consistent with this hypothesis, HP1 γ depleted h*FXN* transgenic YG8 mouse upregulated *FXN* expression [Figure 2.14]. An alternative explanation for this upregulation is given in discussion 2.3.3. It should be noted that, a mouse model that does have a non-pathological h*FXN* without the repeat expansion locus should be evaluated in order to strengthen this finding. Such mice have been recently generated by Mark Pook (Brunel University London, personal communication). Also, RNAi knockdown of HP1 γ as well as other heterochromatic factors using *in vitro* FRDA cells would be a beneficial approach in order to address the direct effect of them on the *FXN* locus.

Has the *FXN* locus evolved a mechanism against the pervasive influence of heterochromatin nucleated by the (GAA) $_n$ repeats? This seems likely as classic chromatin insulator protein CTCF as well as subunits of cohesin ring were found to be enriched in Ex1, where heterochromatin marks are mostly diminished and RNAPII accumulates [Figure 2.4 and 2.9]. Although De Biase *et al.* (2009) previously reported less CTCF binding in a region close to Ex1 in FRDA cells, results presented here could not confirm such difference. Interestingly though, CTCF and cohesin binding was slightly higher in the Int1P4 region in the presence of expanded (GAA) $_n$ repeats. The fact that chromatin insulators and cohesins are involved in the maintenance of the higher order organisation of chromatin [see general introduction 1.5.4] suggests the possibility that the pathological *FXN* locus interacts with other genomic regions. One may expect that such higher order organisation may cause the *FXN* locus to be influenced by the chromatin environment of its interacting regions as suggested in general

by genome-wide studies (Cuddapah *et al.*, 2009; Guelen *et al.*, 2008). Indeed, circular chromosome conformation capture (4C) data generated by Jackson P.K. Chan, Richard Festenstein and MRC CSC Genomic Centre showed that such interactions take place at the *FXN* locus [MRC CSC-London, data not shown, unpublished]. This study also revealed increased intra-regional interactions on the *FXN* locus in the presence of expanded (GAA)_n repeats. In order to unravel the role of CTCF on the *FXN* locus, CTCF binding sites could be mutated by site directed mutagenesis and its effect on *FXN* expression should be tested further.

2.3.2 Upregulating *FXN* expression with the sirtuin inhibitor nicotinamide

Consistent with the idea that HDAC inhibitors can reverse heterochromatin-mediated gene silencing (review-Festenstein, 2006), findings here and in Nadine Rothe's thesis revealed that HDAC class III (Sirtuin) inhibitor nicotinamide, which is found naturally in the human body and is considered as a food supplement [see general introduction 1.3.2.4], can upregulate *FXN* to potentially therapeutic levels. The effect of nicotinamide here was shown *in vitro* (EBV-transformed lymphoblastoid cells) *ex vivo* (primary lymphocytes) and *in vivo*, (YG8 h*FXN* transgenic mouse model) as shown in figure 2.5. Crucially, nicotinamide injection enhanced *FXN* expression significantly in the nerve tissues (i.e. forebrain, cerebellum and spinal cord) of the FRDA mouse model, implying a potentially therapeutic effect is indeed possible within tissues implicated in the pathogenesis of FRDA. Interestingly, *in vitro* and *ex vivo* studies revealed a slight downregulation at the level of protein in healthy cells as opposed to the significant upregulation in the FRDA cells. Notably, other studies investigating the effect of HDAC inhibitors on *FXN* expression did not address protein levels in the healthy cells so far, although they reported upregulation in FRDA cells. It is tempting to speculate that the effect observed here could be explained by the fact that *FXN* protein might be toxic when present at high levels. Although a previous study reported that *FXN* over-expressing mice did not exhibit significant toxicity symptoms (Miranda *et al.*, 2004), other studies with human fibroblasts and *Drosophila* concluded that elevated levels of *FXN* protein can indeed be toxic and have functionally deleterious effects (Fleming *et al.*, 2005b; Navarro *et al.*, 2011). Therefore, downregulation of *FXN* protein in the healthy cell line could be a protective response in order to prevent such negative effects. Of course this speculation is based upon a limited data set and further experiments are needed to establish the downregulation of *FXN* protein in healthy cells.

How does nicotinamide upregulate the pathologically silenced *FXN* gene? Nicotinamide is a classical sirtuin inhibitor whose primary targets are SIRT1 and SIRT2 [see general introduction 1.3.2.4]. Both of these enzymes are known to have histone deacetylase

activities particularly at H4K16 and H3K9 residues (Vaquero *et al.*, 2004; Vaquero *et al.*, 2006). Several attempts were made in order to see if the acetylation levels of these specific residues are increased by nicotinamide treatment; however, no such effect was observed yet [data not shown]. Although this experiment might be worth repeating with other antibodies, it seems more likely that nicotinamide exerts its upregulating function in a different mechanism at the *FXN* locus. Interestingly, heterochromatin marks H3K9me3 and H3K27me3 are diminished upon nicotinamide treatment in *ex vivo* primary cells and in the implicated tissues of the FRDA mouse model [Figures 2.6 and 2.7]. Consistently, overall acetylation levels of histones H3 and H4 were increased [Figure 2.8]. Also, preliminary results by Raul Torres (MRC CSC, London) suggested an increase in H3K9 acetylation levels in Ex 1 [data not shown]. Moreover, increased DNase-I hypersensitivity of the pathological *FXN* locus upon nicotinamide was reported by Jackson P.K. Chan [MRC CSC-London, unpublished data]. These results suggest that nicotinamide has the ability to reverse the abnormally induced heterochromatin in the case of expanded (GAA)_n repeats. It is intriguing to note that the primary nicotinamide target SIRT1 activates a key heterochromatin inducer SUV39H, which catalyses the tri-methylation of H3K9 residues (Vaquero *et al.*, 2007). Consistent with this, SIRT1 knock-down by siRNAs resulted in a generalised decrease in H3K9me3 in human cells (Vaquero *et al.*, 2004). Preliminary results by Pui-Pik Law (MRC CSC, London) showed SIRT1 binding on the *FXN* locus [data not shown]. It is also worth investigating the binding of SUV39H on the *FXN* locus, although no ChIP compatible antibodies for this factor have been revealed yet. In order to address whether inhibition of SUV39H indeed upregulates *FXN*, one may consider inhibiting this enzyme chemically (Illner *et al.*, 2010) or by RNAi approaches.

In addition to its ability to upregulate *FXN* expression, nicotinamide may also have secondary beneficial effects in FRDA patients. Interestingly, nicotinamide is known to be a common agent to relieve cellular symptoms of oxidative stress, which is thought to be one of the reasons behind the progression of the FRDA phenotype [see introduction 2.1.3] (Crowley *et al.*, 2000; Li *et al.*, 2006a; review-Lin *et al.*, 2000). This further strengthens the therapeutic potential of nicotinamide. The exact effect of nicotinamide on oxidative stress mechanisms is not currently well known, although they were not linked to *FXN* so far [see general introduction 1.3.2.4]. Another intriguing point to mention is that nicotinamide may have the potential to inhibit the activity of PPAR γ , which is thought to upregulate *FXN* expression [see introduction 2.1.5]. PPAR γ operates with its co-activators PGC1 α and β , whose activation is dependent on the deacetylation activity of SIRT1 (Rodgers *et al.*, 2005). Thus, PPAR γ 's effect in upregulating *FXN* would be inhibited by nicotinamide. However, results presented in this chapter showed a significant upregulation of *FXN* expression by nicotinamide; which

therefore implies that whatever the effect on PPAR γ the upregulatory effect of nicotinamide on *FXN* expression is dominant.

Although findings here strongly suggest a therapeutic effect of nicotinamide *in vivo* using a mouse model, this should be tested further on humans. Due to its low potential to cause side effects and its bioavailability, it is anticipated that nicotinamide is likely to reach clinical use for the FRDA treatment. Still, *in vitro* and *ex vivo* wash-out experiments with FRDA cell lines as well as further time course experiments with mouse models would give beneficial information regarding to the duration of the upregulating effect of nicotinamide on the silenced *FXN* locus. Also, the physical activity and liver enzymatic profile of such mice should be evaluated further.

2.3.3 RNAPII stalling on *FXN* and its dysregulation by the proteasome

Another key finding investigated in this chapter is the RNAPII stalling site on the *FXN* gene. How high levels of heterochromatin or unusual DNA structures formed by the expanded (GAA)_n repeats [see introduction 2.1.6] interfere with the RNAPII transcription has been an important research question in the FRDA field. Here, RNAPII was shown to accumulate in Ex1 where heterochromatin marks are diminished and acetylation is increased [Figures 2.3, 2.8 and 2.9]. Interestingly, H3K4me2 which correlates with recently transcribed chromatin [see general introduction 1.3.3.2 and 1.6.3.2], is also enriched in this region; however, no difference was observed between the healthy and FRDA cells [Figure 2.9]. Also, there was no decrease in both FRDA cell lines in the initiating (S5p) and elongating (S2p) forms of RNAPII levels at this region. This suggests that the silencing of *FXN* in FRDA could not be explained by reduced RNAPII or an initiation defect. Consistent with this, Punga *et al.* (2010) reported no significant differences in H3K4 methylation profiles immediately upstream of (GAA)_n and reduced H3K36 methylation levels in FRDA cells along the gene body. Taken together with similar levels of S5p RNAPII levels upstream of (GAA)_n, Punga *et al.* (2010) concluded that a transcriptional elongation defect is the major problem in FRDA. Moreover, Kim *et al.* (2011) reported reduced levels of *FXN* primary transcripts only downstream of (GAA)_n. Using an antibody that does not distinguish phosphorylated forms, they did a RNAPII ChIP and reported reduced RNAPII levels in FRDA immediately upstream of (GAA)_n but not at the gene promoter. They also demonstrated reduced H3K36 as well as H3K79 methylation levels downstream of (GAA)_n. On the other hand, another study performed by Kumari *et al.*, suggested that both initiation and elongation is affected in FRDA, based on ChIP assays with antibodies that distinguish S5p and S2p phosphorylated forms of RNAPII. However, the antibodies used in this study were found to be confounded due to the fact that

they were cross reacting with the wrong serine phosphorylation status [personal communication with Emily Brookes and Ana Pombo, MRC CSC-London].

Here, ChIPs after transcriptional blocking as well as RNA immunoprecipitation revealed that the RNAPII within Ex1 is stalling in healthy cells [Figures 2.10 and 2.11]. Given the fact that it has been suggested that the *FXN* gene is involved in stress response mechanisms [see introduction 2.1.3], it is possible that such a stalling effect could be advantageous in terms of preparing a ready-to-go reservoir of RNAPII complexes in order to upregulate the gene rapidly upon stimuli. Such 'pausing' effects are known to take place in the promoter-proximal regions of the heat shock response gene *Hsp70* (Brown *et al.*, 1996; Gilmour and Lis, 1986; Sun and Li, 2010), oncogenes *c-MYC* (Eick *et al.*, 1987) and *P21* (Espinosa *et al.*, 2003) as well as latent phase HIV-LTR (Kao *et al.*, 1987). Considering the potential roles of *FXN* protein on iron metabolism and oxidative stress, one may consider analysing the levels of RNAPII stalling via ChIP after treating FRDA cells *in vitro* or *ex vivo* with excess iron or agents that induce oxidative stress (e.g. hydrogen peroxide).

What is unusual about the newly identified RNAPII stalling site on the *FXN* gene is the fact that both initiating (S2p) and elongating (S5p) forms of RNAPII are affected. Importantly, this was not shown before for the promoter-proximal paused genes (review-Brookes and Pombo, 2009) [see general introduction figure 1.18]. Therefore, the stalling effect described here seems more analogous to the stalling of RNAPII when it encounters a damaged DNA lesion and initiates the transcription-coupled repair response (review-Hanawalt and Spivak, 2008; Mellon *et al.*, 1987; review-Svejstrup, 2003). In the case of *FXN*, the stalling may take place due to the physical blocking effect of unusual DNA structures formed by the (GAA)_n repeats [see introduction 2.1.6]. Indeed, hairpins or loops created by (CNG)_n and (GAA)_n repeats were found to cause RNAPII arrest as shown by *in vitro* assays (Grabczyk *et al.*, 2007; Salinas-Rios *et al.*, 2011). Moreover, the heterochromatin induced by (GAA)_n repeats could theoretically cause this stalling effect, although apparently no such observation has been made before. Are there specific factors that promote RNAPII stalling at the *FXN* locus? This needs to be further investigated; however, it is intriguing to note that CTCF, which is present at the stalling site, has recently been linked to RNAPII pausing, although the mechanism has not been identified yet (Shukla *et al.*, 2011). Mutating CTCF binding sites on the *FXN* locus would therefore give information about the effect of CTCF on the RNAPII stalling. The key discovery presented here is that the stalled RNAPII is subject to proteasomal degradation in the presence of expanded (GAA)_n repeats, as RNAPII levels followed by transcriptional inhibition could be restored in the presence of proteasome inhibitors [Figures 2.12 and 2.13]. Indeed, persistently stalled RNAPII-S2p is known to be targeted by the proteasome in

transcription coupled repair (Anindya *et al.*, 2007; Bregman *et al.*, 1996; Lee *et al.*, 2002; Mitsui and Sharp, 1999; Somesh *et al.*, 2005; Somesh *et al.*, 2007; Woudstra *et al.*, 2002). Here, stalled RNAPII-S2p in healthy cells seems to escape proteasomal degradation perhaps by adopting an unusual conformation that was not documented before. The stalled RNAPII in healthy is thought to be slowly released from Ex1 giving rise to *FXN* expression [Figure 2.15 upper panel]. This stalling site could be a secondary transcriptional check point to maintain physiologically low levels of *FXN* expression (Campuzano *et al.*, 1997; Su *et al.*, 2004), because high levels of *FXN* protein under normal conditions may be toxic (Fleming *et al.*, 2005b; Navarro *et al.*, 2011). Susceptibility of RNAPII-S2p to proteasomal degradation in FRDA cells is thought to be increased by the elevated levels of the proteasome on the pathologically silenced *FXN* locus [Figure 2.12A and supplementary figure S.8]. Interestingly, proteasome enrichment patterns reflected those of heterochromatin marks and HP1 β , supporting the notion that expanded (GAA)_n repeats may induce the recruitment of the proteasome. Therefore, the proteasome is thought to provide a new dimension for the heterochromatic gene silencing to take place in the presence of expanded (GAA)_n repeats [Figure 2.15 middle panel]. Indeed, proteasome inhibition upregulated *FXN* expression significantly [Figure 2.12, 2.13 and supplementary figure S.9] and thereby provided a novel therapeutic avenue against FRDA [Figure 2.15 lower panel]. Still, the effects of proteasome inhibition should be tested on the YG8 FRDA mouse model by injecting mice in a carefully planned time schedule. Also, more experiments with the *ex vivo* primary cells would strengthen the results presented here.

How the expanded (GAA)_n repeats induce the recruitment of the proteasome remains to be investigated further. Interestingly, (GAA)_n repeats are known to be occupied by mismatch repair proteins such as MSH2 (Bourn *et al.*, 2009; Ku *et al.*, 2010), which were shown to interact with proteasomal subunits (Hernandez-Pigeon *et al.*, 2004). Moreover, HP1 γ enrichment was detected at the latent phase HIV-LTR, where RNAPII pausing also takes place (du Chene *et al.*, 2007; Kao *et al.*, 1987). It was also realised that HP1 γ binding at the latent phase HIV-LTR, correlates well with the recruitment of the proteasome which is thought to silence the viral gene (du Chene *et al.*, 2007; Lassot *et al.*, 2007). Moreover, HP1 γ was shown to co-immunoprecipitate with the regulatory particles of the proteasome by another independent study (Vermeulen *et al.*, 2010). Consistent with these, HP1 γ is enriched in Ex1 of the *FXN* locus, where RNAPII stalling occurs [Figure 2.3]. This correlation indeed suggests that HP1 γ may play a role in regulating this RNAPII stalling site. Although HP1 γ binding was not increased in FRDA cells compared to healthy, it is consistently enriched on the stalling site [Figure 2.3] and therefore might function to increase the susceptibility to degradation by the proteasome, which is already present at higher levels in the presence of

expanded (GAA)_n repeats [Figure 2.12]. Also, it is known that HP1 proteins can form heterodimers (review-Hiragami and Festenstein, 2005) and therefore it is likely that HP1 γ forms heterodimers with HP1 β which is found at higher levels on the silenced *FXN* locus. In line with this; HP1 γ depletion upregulated *FXN in vivo* [Figure 2.14], although the direct effect of this depletion on RNAPII stalling should be measured further by ChIP. Also, it would be wise to check the HP1 γ levels on humanised h*FXN* mice without the repeat expansion.

As heterochromatin at the silenced *FXN* locus appears to recruit the proteasome and plays a role in gene silencing by degrading RNAPII this suggested the possibility that heterochromatin per-se may also be regulated by a similar mechanism. This idea is pursued throughout the next chapter.

Figure 2.15

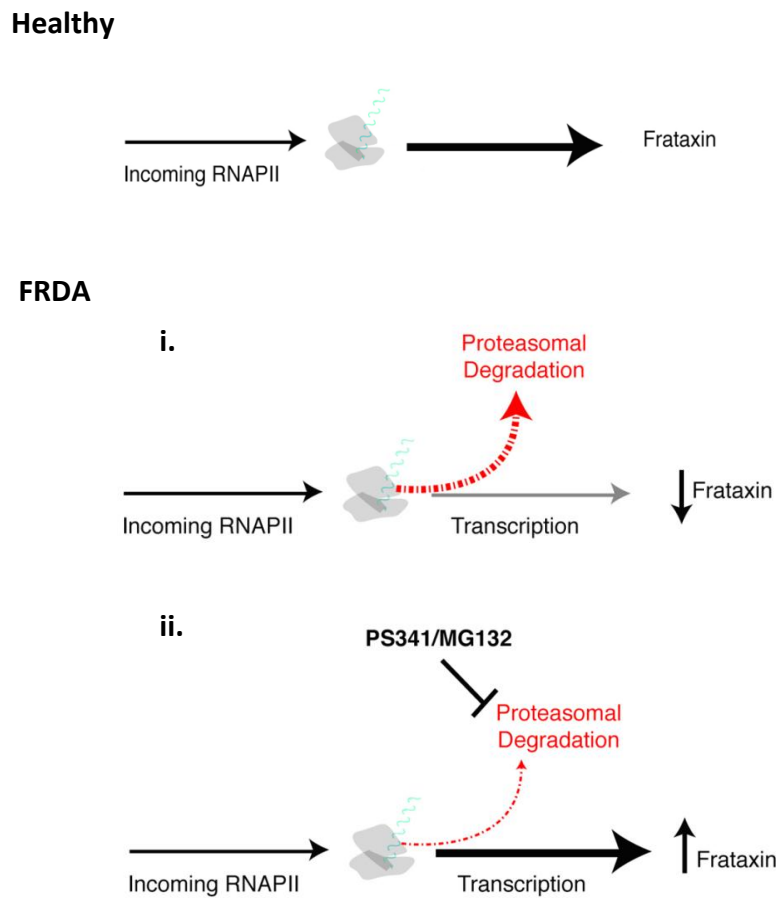


Figure 2.15 – An illustrative model representing the effect of proteasome inhibition on FRDA.

In this thesis, ChIP results and transcription blocking experiments suggest that RNAPII is stalled within the first exon of *FXN*. This may have functional significance in terms of rapidly upregulating the gene in response to a signal. Also it could be a secondary check-point for the gene to be expressed at low levels under normal conditions, as high levels of Frataxin can be toxic. In the absence of expanded (GAA)_n repeats, RNAPII is thought to be released from the stall site, giving rise to *FXN* expression [upper panel]. In the presence of expanded (GAA)_n repeats, there is an increased tendency for the proteasome to degrade stalled RNAPII complexes, preventing efficient transcription rates to take place [middle panel,ii]. However, inhibiting the proteasome by chemical inhibitors such as PS341 or MG132 upregulates the gene up to potentially therapeutic levels by restoring the reservoir of RNAPII complexes. Overall, results here opened a new therapeutic avenue against FRDA.

2.4 Contributions

Nicotinamide was first shown to upregulate *FXN* by Nadine Rothe. ChIP with primary lymphocytes following nicotinamide treatment were performed by Cihangir Yandim, Raul Torres and Jackson P.K. Chan collaboratively (equal contributions).

RNAPII stalling site was first shown by Maria Mayan and Nadine Rothe. ChIP experiments with transcription blockers were performed collaboratively by Cihangir Yandim, Maria Mayan, Raul Torres and Jackson P.K. Chan (equal contributions).

Everything else presented in this chapter was performed by Cihangir Yandim.

CHAPTER 3

A POTENTIAL LINK BETWEEN HETEROCHROMATIN AND THE PROTEASOME

3.1 Introduction

3.1.1 Chromatin related functions of the proteasome and gene regulation

Although the proteasome was originally found to function within the cytoplasm, growing evidence now also suggests essential nuclear roles for the proteasome holoenzyme or its individual components. Nuclear localisation of the proteasome, whose components are imported from the cytoplasm mostly by karyopherin $\alpha\beta$ at least in yeast, seems to depend on the cell type or environmental stimuli (Lehmann *et al.*, 2002; Wendler *et al.*, 2004). Specifically, the proteasome has been detected in mammalian cell nuclei within the PML (promyelocytic leukemia protein) nuclear bodies (Fabunmi *et al.*, 2001), nucleoplasmic speckles (Chen *et al.*, 2002), focal nuclear clusters (Rockel *et al.*, 2005) or at the nuclear envelope (Feist *et al.*, 2007).

Just like in the cytoplasm, the proteasome is involved in the 'quality control' of proteins inside the nucleus (Schubert *et al.*, 2000). A nuclear quality control system was identified in *S. cerevisiae*, where a specific ubiquitin ligase targets mutant or misfolded nuclear proteins (Gardner *et al.*, 2005). Moreover, the proteasome also seems to play a role in DNA replication as inhibiting proteasome function has a suppressing effect on DNA replication of human cytomegalovirus (Kaspari *et al.*, 2008) or genomic replication of *Toxoplasma gondii* (Shaw *et al.*, 2000). Similar effects were seen in the replication of herpes simplex virus (HSV) and human immunodeficiency virus (HIV) (Eom and Lehman, 2003; Li *et al.*, 2010a). Moreover, the large subunit of the human origin recognition complex (ORC1) is degraded by the proteasome after the initiation of DNA replication providing a crucial step for later events in replication (Mendez *et al.*, 2002).

Perhaps one of the strongest links between the proteasome and chromatin is the regulation of DNA-damage response mechanisms. For instance, the yeast nucleotide-excision repair (NER) protein RAD23 interacts with the proteasome (Schauber *et al.*, 1998). This interaction seems to have a regulatory role on NER due to the 19S ATPases rather than a proteolytic effect (Russell *et al.*, 1999). Likewise, studies from yeast and mammals suggest a regulatory function for 19S on homologous recombination (HR) and non-homologous end joining (NHEJ) repair based on interactions with DSS1 (deleted in split hand/split foot) and BRCA2 (breast cancer 2) (Gudmundsdottir *et al.*, 2007; Krogan *et al.*, 2004). There is also evidence suggesting the recruitment of the mammalian 11S regulatory particle to the damaged lesions (Ustrell *et al.*, 2002). The role of regulatory proteasome particles in DNA repair context is not well understood yet but it has been proposed that these complexes potentially have remodeling activity which is necessary for the stepwise events during the repair process

(review-Ramadan and Meerang, 2011; Russell *et al.*, 1999). In addition to those described so far, proteolytic function of the proteasome is also important in DNA repair. For example, proteasome-dependent turnover of a yeast double-strand break protein MMS22 is essential in terms of cell cycle progression and chromosome stabilisation after DNA repair (Ben-Aroya *et al.*, 2010). Similarly, MSH2/MSH6 heterodimers, which are essential components of the mismatch repair machinery, are recognised by the proteasome and degraded after completing their task on damaged DNA (Hernandez-Pigeon *et al.*, 2004). Another interesting finding is the direct stimulatory effect of poly(A)-ribosylation (a hallmark of DNA damage response) and its responsible enzyme PARP1 on the proteolytic activity of the 20S proteasome (Mayer-Kuckuk *et al.*, 1999). This was shown to induce chromatin repair after oxidative damage by replacing damaged histones (Catalgol *et al.*, 2010). Finally; as explained in the general introduction 1.6.4, a persistently stalled RNAPII in transcription-coupled repair is degraded by the 26S proteasome (Mitsui and Sharp, 1999; Somesh *et al.*, 2005; Woudstra *et al.*, 2002). In line with these observations, proteasome inhibition results in perturbation of DNA repair pathways and enhanced sensitivity to damage-inducing agents (Takeshita *et al.*, 2009).

Close association of the proteasome with chromatin is also exemplified by its effects on transcription (review-Kwak *et al.*, 2011). Because the proteasome participates in a large number of cellular pathways, it is often difficult to distinguish its direct effects on transcription. Still, a number of genome-wide studies implied an endogenous role for the proteasome in transcription as its inhibition by genetic, RNAi or chemical methods influenced the expression patterns of a large genome fraction in yeast and human cells (Dembla-Rajpal *et al.*, 2004; Fleming *et al.*, 2002; Tang *et al.*, 2008; Zimmermann *et al.*, 2000). Furthermore, two genome-wide studies carried out with *S. cerevisiae* were able to strengthen the proteasome-transcription link by combining chromatin immunoprecipitation with microarrays (Auld *et al.*, 2006; Sikder *et al.*, 2006).

In yeast, the 19S particle is known to assist various non-proteolytic activities in transcriptional regulation. For instance, efficient gene activation by yeast transcriptional activator GAL4p is linked to ATPase activities of 19S subunits, which can modulate the activator's stability in *S. cerevisiae* (Gonzalez *et al.*, 2002; Nalley *et al.*, 2006). A chaperone-like activity of 19S was also reported within the GAL4 system, where 19S directly recruits co-activator complex SAGA (Lee *et al.*, 2005a; Malik *et al.*, 2009). Moreover, 19S was shown to indirectly contribute to chromatin disassembly events with the FACT complex during the transcriptional induction of the yeast *PHO5* promoter (Ransom *et al.*, 2009). Although this study ruled out the possibility of direct chromatin remodeling activity of 19S, such functions

could be possible in broader contexts (review-Kwak *et al.*, 2011). This was strengthened by another finding that 19S co-immunoprecipitates with the yeast transcription elongation factor CDC68; also 19S mutants show elongation defects whereas 20S mutants do not (Ferdous *et al.*, 2001). In the presence of H2B1 monoubiquitination (a mark for transcription elongation), 19S assists functions of yeast COMPASS complex and DOT1 methylase, participating in the elongation related H3K4 and subsequent H3K79 methylations respectively (Dover *et al.*, 2002; Ezhkova and Tansey, 2004; Laribee *et al.*, 2007; Lee *et al.*, 2007a). Moreover, 19S also seems to promote H3 acetylation on yeast reporter genes (Koues *et al.*, 2008). Although some attempts were made, it is still unclear if these transcription-related functions of 19S take place in mammals (Fatyol and Grummt, 2008; Ferdous *et al.*, 2002). Still, the 19S proteasome could exert different roles when it is associated with the catalytic 20S particle, which needs at least a regulatory particle in order to function [see general introduction 1.7.1]. Finally, the proteolytic role of the 26S proteasome in yeast transcription primarily involves mediating the termination of RNAPII termination, where it facilitates the release of RNAPII from the 3' end of the coding sequence (Gillette *et al.*, 2004).

It was realised that many studies on proteasome function in *S. cerevisiae* focused on the 19S subunit and these were not successfully reproduced within the cellular context in mammals. Interestingly, a number of studies with mammalian cells suggest transcription-limiting functions for the 26S proteasome holoenzyme. For example, recycling of nuclear receptor activators such as estrogen, glucocorticoid, progesterone, thyroid hormone or retinoic acid receptors by 26S proteasome were shown to limit the transcription rate and ensure a moderate level but continuous transcription of target genes as a response to hormones (review-Keppler *et al.*, 2011; review-Nawaz and O'Malley, 2004). However the mechanistic links between the target gene promoter and the proteasome recruitment remains unclear within this context (review-Keppler *et al.*, 2011). Overall, these studies have implications regarding to transcription inhibiting functions of the proteasome and these will be explained in detail in the discussion section. Consistently, results from the previous chapter highlighted a role for the proteasome in the transcriptional regulation of the human *FXN* gene, which is heterochromatinised in the case of expanded (GAA)_n repeats and gives rise to Friedreich's ataxia. Here, it was identified that the proteasome is degrading RNAPII and thereby limiting transcription in the heterochromatinised *FXN* gene. Crucially, binding of the proteasome was generally higher along the silenced *FXN* gene, correlating well with the heterochromatic H3K9me3 mark. This suggested a synergistic link between heterochromatin and the proteasome in relation to transcriptional silencing.

3.1.2 Hypothesis

So far, heterochromatic gene silencing has been thought to occur only due to the restricted access of RNAPII because of an inaccessible chromatin structure. Based on the finding on the human *FXN* gene, it was hypothesised that the proteasome might provide an unidentified dimension of mammalian heterochromatic gene silencing by actually degrading RNAPII and hence lowering the chances of transcription to occur.

3.2 Results

3.2.1 Proteasome binding is enriched at heterochromatin in the EBV-transformed human lymphoblastoid cell line GM14926

Chromatin immunoprecipitation (ChIP) experiments at the human *FXN* locus revealed a link between heterochromatic gene silencing and the proteasome in the EBV-transformed lymphoblastoid cell lines as well as primary lymphocytes. The obvious question to ask was whether this was limited to the *FXN* locus or was it a specific example of a more generalised phenomenon. Therefore, it was decided to use ChIP against the proteasome on other loci using the 'healthy' GM14926 cell line which served as a 'normal' control for many experiments in the previous chapter. Here, ChIP was performed using antibodies against RNAPII (S5p), H3K9me3 and the 19S proteasome (RPN10) subunit [Figure 3.1], in a similar way with what was presented in the previous chapter.

In order to analyse the link between the chromatin status and proteasome binding, ChIP material was quantified with Q-RT-PCR using primers that amplify various loci which represent different types of chromatin. Accordingly, highly heterochromatic status was represented by centromeric α -satellites and pericentromeric γ - and SN5 satellites [see introduction 1.8.3.2]. Because transposable elements were shown to be subject to heterochromatinisation at various occasions [see general introduction 1.8.2.2 and 4.3.4], they were also of special interest within the context of this thesis. Therefore, the two most common transposons, LINE (L1) and SINE (Alu) elements were amplified. In addition, a similar analysis was performed on several inactive genes, in order to determine whether similar heterochromatic factors might play a gene silencing role. These include the erythroblast specific β -globin (beta haemoglobin), brain-specific *MOG* (myelin oligodendrocyte glycoprotein) and liver-specific *HP* (haptoglobin) which were selected according to TiGER [<http://bioinfo.wilmer.jhu.edu/tiger/>]; a database for tissue specific gene expression (Liu *et al.*, 2008). On the other hand, three housekeeping genes; *HMBS* (hydroxymethylbilane synthase), *HPRT* (hypoxanthine phosphoribosyltransferase) and *ALDOA* (aldolase A) represented transcriptionally active euchromatic locations. Because the effect of proteasome on RNAPII was obvious within the first exon of the human *FXN* gene, primers both for inactive and active genes were designed so that their first exons are amplified.

As expected, analysis of co-immunoprecipitated DNA revealed that RNAPII levels are much lower both on the repeat elements [Figure 3.1A, black] and inactive genes [Figure 3.1A, grey]. However, actively expressed housekeeping genes displayed a high level of RNAPII

binding [Figure 3.1A, white]. On the contrary, the heterochromatic mark H3K9me3, which is often associated with transcriptional repression, was highly enriched on repeat elements as previously shown (Martens et al, 2005) and was reduced in housekeeping genes [Figure 3.B]. Notably, the extent of H3K9me3 was highest in pericentromeric γ -Sat and SN5 repeats, whereas it was slightly lower in centromeric repeats. This could be explained by the fact that centromeric DNA is folded around CENP histones but not H3 [see general introduction 1.2.4]. It is also interesting to note that LINE (L1) elements exhibited H3K9me3 levels to an extent close to inactive genes, corresponding well with previous observations that LINE elements were detected in silenced gene loci in human cells (Eller *et al.*, 2007; Ganapathi *et al.*, 2005; Han *et al.*, 2004).

Consistent with the *FXN* result in the preceding chapter [Figures 2.3 and 2.12], 19S proteasome binding correlated well with H3K9me3. It was found to be significantly higher in repeat elements and inactive genes and much lower in the housekeeping genes. This result with the 19S regulatory particle independently confirms that there is indeed a link between heterochromatin and the proteasome in the GM14926 human lymphoblastoid cell line. Therefore, the effect seen on *FXN* is highly likely to be a result of heterochromatinisation of the gene in the presence of (GAA)_n repeats, rather than a (GAA)_n specific mechanism.

Figure 3.1

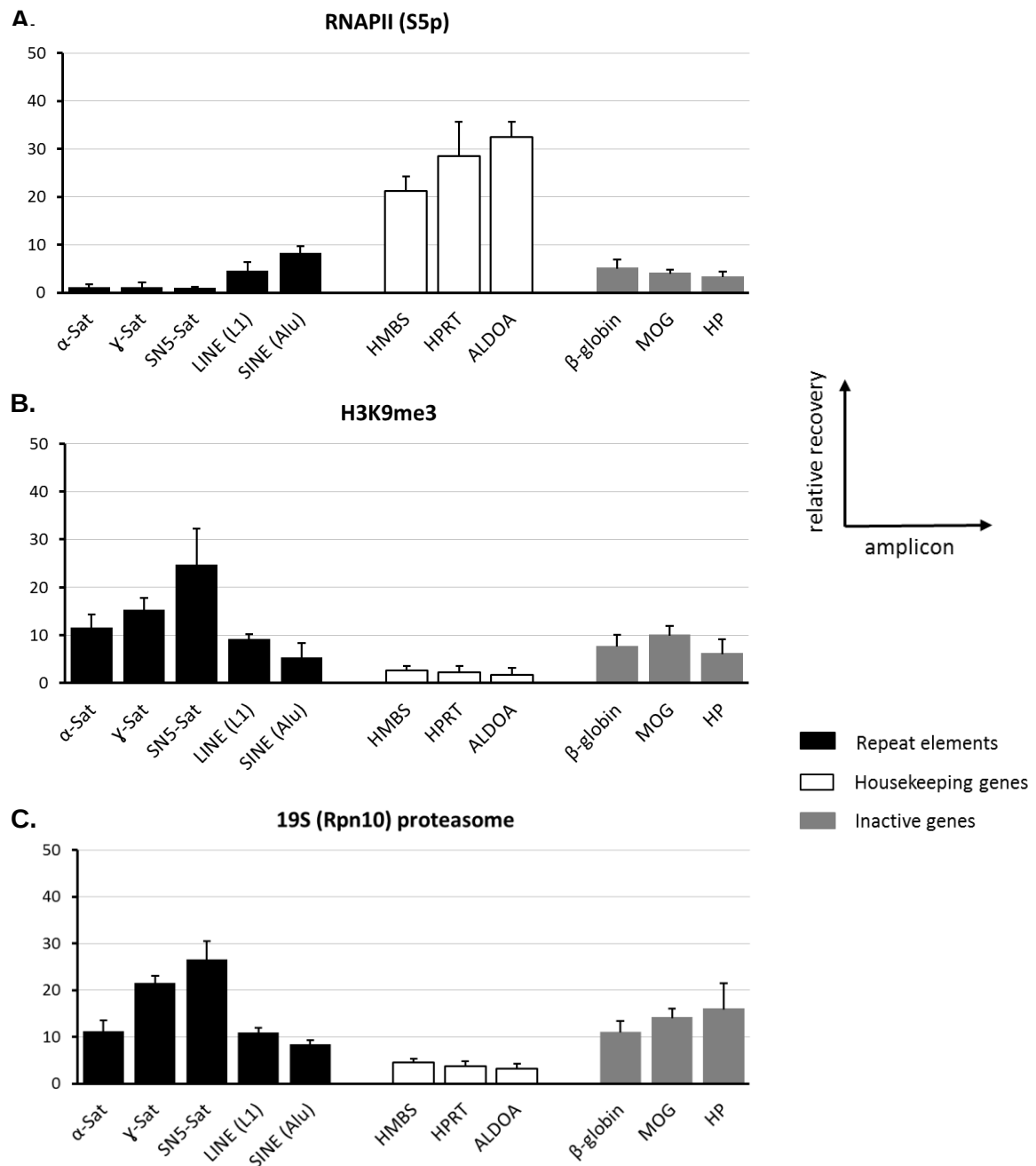


Figure 3.1 – ChIP against RNAPII, H3K9me3 and 19S proteasome with the human GM14926 cell line. [A] **Enrichment of RNAPII (S5p).** RNAPII levels are much higher in housekeeping genes than heterochromatic repeat elements or inactive genes. [B] **Enrichment of H3K9me3.** In contrast to RNAPII, levels of the heterochromatic mark H3K9me3 were much higher in repeat elements and inactive genes. [C] **Enrichment of the 19S (Rpn10) proteasome.** Correlating well with the H3K9me3 levels, 19 S proteasome binding was higher in repeat elements and inactive genes. [Enrichment levels are presented as percentage to input DNA. Error bars represent the SEM value of three independent experiments.]

3.2.2 Proteasome binding is enriched in heterochromatin at mouse thymus

Results with the immortalised human cell line uncovered a more general link between heterochromatin and the proteasome. In order to address whether this also takes place *in vivo*, further experiments were performed on mouse thymus. A lot of work in relation to other aspects of chromatin had already been performed on mouse thymus in Richard Festenstein's laboratory and therefore it would be easier to compare the outcome of experiments. Also, mouse thymus is advantageous in terms of the fact that it can be easily dissociated to individual cells for immunofluorescence or FACS analysis. Experiments were performed on young 4 weeks old male mice (wild type CBA.ca), because of the well-known fact that thymus tissue atrophies with age (Aspinall, 1997; Scollay *et al.*, 1980).

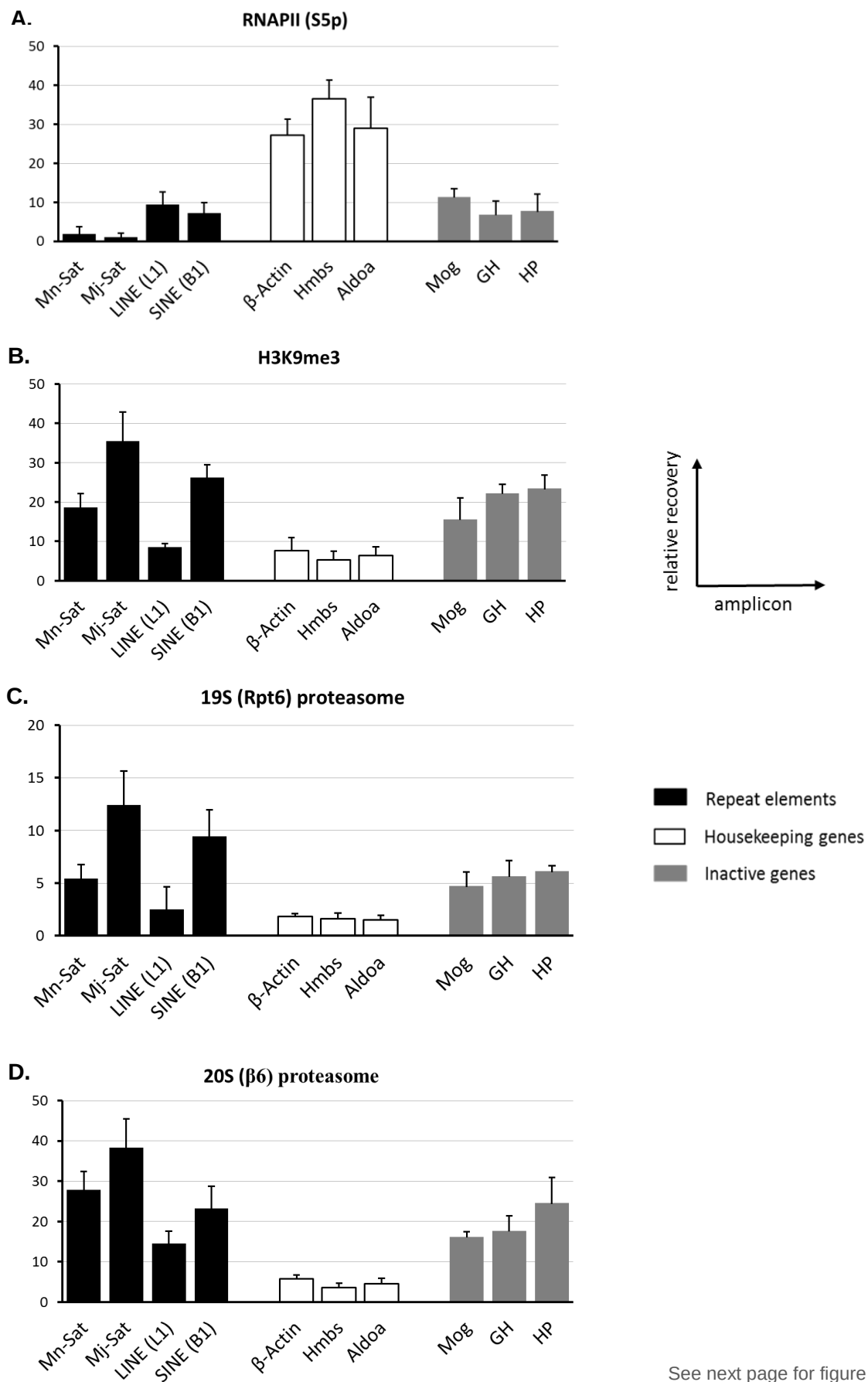
In a similar approach to that explained above, chromatin was immunoprecipitated using antibodies against RNAPII, H3K9me3 as well as the 19S (RPT6) and 20S (β 6) proteasome particles. Proteasome antibodies were screened and several antibodies which recognise either the 19S and 20S proteasome particles were identified. These are presented on the supplementary table S.2. The ones which gave the highest signal:background ratio were used in ChIP assays. Immunoprecipitated material was quantified again using primers that amplify regions that represent different states of chromatin. In order to address highly heterochromatic regions, centromeric minor and pericentromeric major repeats were analysed [see general introduction 1.8.3.2]. Similarly, LINE (L1) and SINE (B1) transposon sequences were of particular interest and were also analysed. To analyse 'silent genes', the first exons of brain-specific *Mog* (myelin oligodendrocyte glycoprotein), brain-specific *GH* (Growth hormone) and liver-specific *HP* (haptoglobin) genes were amplified (TiGER database, see the preceding section). Similarly, housekeeping genes β -actin, *Hmbs* (hydroxymethylbilane synthase) and *Aldoa* (aldolase A) were selected to represent transcriptionally active euchromatin.

According to the Q-RT-PCR analysis, centromeric minor and pericentromeric major repeats as well as SINE (B1) elements were deprived of RNAPII [Figure 3.2A, black] and enriched in the heterochromatic H3K9me3 mark [Figure 3.2B, black]. Also, inactive genes exhibited a low level of RNAPII binding [Figure 3.2A, grey] and a moderately high level of H3K9me3 enrichment [Figure 3.2B, grey], whereas the situation was the opposite for active housekeeping genes [Figures 3.2A and 3.2B, white]. Interestingly, LINE (L1) was found to have a lower level of the heterochromatin mark. This result was discussed further in the next chapter [see section 4.3.4]. Importantly, corresponding well with the human cell line, both 19S and 20S proteasome particles were found enriched at heterochromatic sequences compared to euchromatic housekeeping genes [Figure 3.2C and 3.2D].

To further validate this result, mouse thymocytes obtained from the teased thymus by cell filtration underwent immunofluorescence (IF) analysis. Methanol fixed and permeabilised thymocytes were stained with DAPI [4',6-diamidino-2-phenylindole; stains DNA, Figure 3.3 blue] and H3K9me3 [Figure 3.3, green] as well as the 20S (β 6) proteasome [Figure 3.3, red] antibodies, which were the same antibodies used for ChIP experiments. In order to detect the binding, antibodies were prelabeled with Alexa Fluor® 488 [H3K9me3, green] and 594 [20S proteasome, red]. As expected, DAPI staining clearly showed dense spots, which are results of tightly packed DNA into heterochromatin. The H3K9me3 signal overlapped well with these heterochromatic foci. Notably, the 20S proteasome signal co-localised with the green signal for H3K9me3 at a moderate level, particularly at heterochromatic foci. It is noteworthy that these spots seem to occur frequently close to the nuclear periphery, where they interact with the proteasome (Figure 3.3, middle and right panel). This is consistent with the previous observations regarding to the peripheral localisations of heterochromatic silenced genes (Alcobia *et al.*, 2000; Andrulis *et al.*, 1998; Dietzel *et al.*, 2004; Hiragami-Hamada *et al.*, 2009).

The co-localisation was analysed with WCIF (Wright Cell Imaging Facility) plugin using the ImageJ software [Rasband, W.S., National Institutes of Health, Maryland, USA, <http://rsbweb.nih.gov/ij/>] as presented in a previous study (Li *et al.*, 2004). A moderate level of correlation ($R^2=0.61$) was calculated with this approach on a cell population of 50. Here, cells were permeabilised with methanol in the IF technique [see materials and methods 6.14]. Methanol is a strong permeabilisation agent and it was used here because it helps for the better intrusion of antibodies into tightly packed heterochromatic regions. However by doing so, it may also affect antibody binding efficiency by disrupting the epitopes. Therefore the correlation between these two antibodies could actually be even higher. Still, ChIP and IF data on mouse thymus revealed that heterochromatin and the proteasome are indeed somehow linked to each other *in vivo*.

Figure 3.2



See next page for figure legend.

Figure 3.2 – ChIP with mouse thymus against RNAPII, H3K9me3 and the proteasome particles.

[A] Enrichment of RNAPII (S5p). RNAPII levels are much higher at housekeeping genes than heterochromatic repeat elements or inactive genes. **[B] Enrichment of H3K9me3.** In contrast to RNAPII, levels of heterochromatic mark H3K9me3 was much higher in repeat elements (except L1) and inactive genes. **[C] Enrichment of 19S (Rpt6) proteasome.** Correlating well with the H3K9me3 levels, 19 S proteasome binding was higher in repeat elements and inactive genes. **[D] Enrichment of 20S (β 6) proteasome.** 20S core particle binding showed similar levels to that of 19S [Enrichment levels are presented as percentage to input DNA. Error bars represent the SEM value of three independent experiments performed on the materials obtained from three different age and sex matched mice, which are ~4 weeks old.]

Figure 3.3

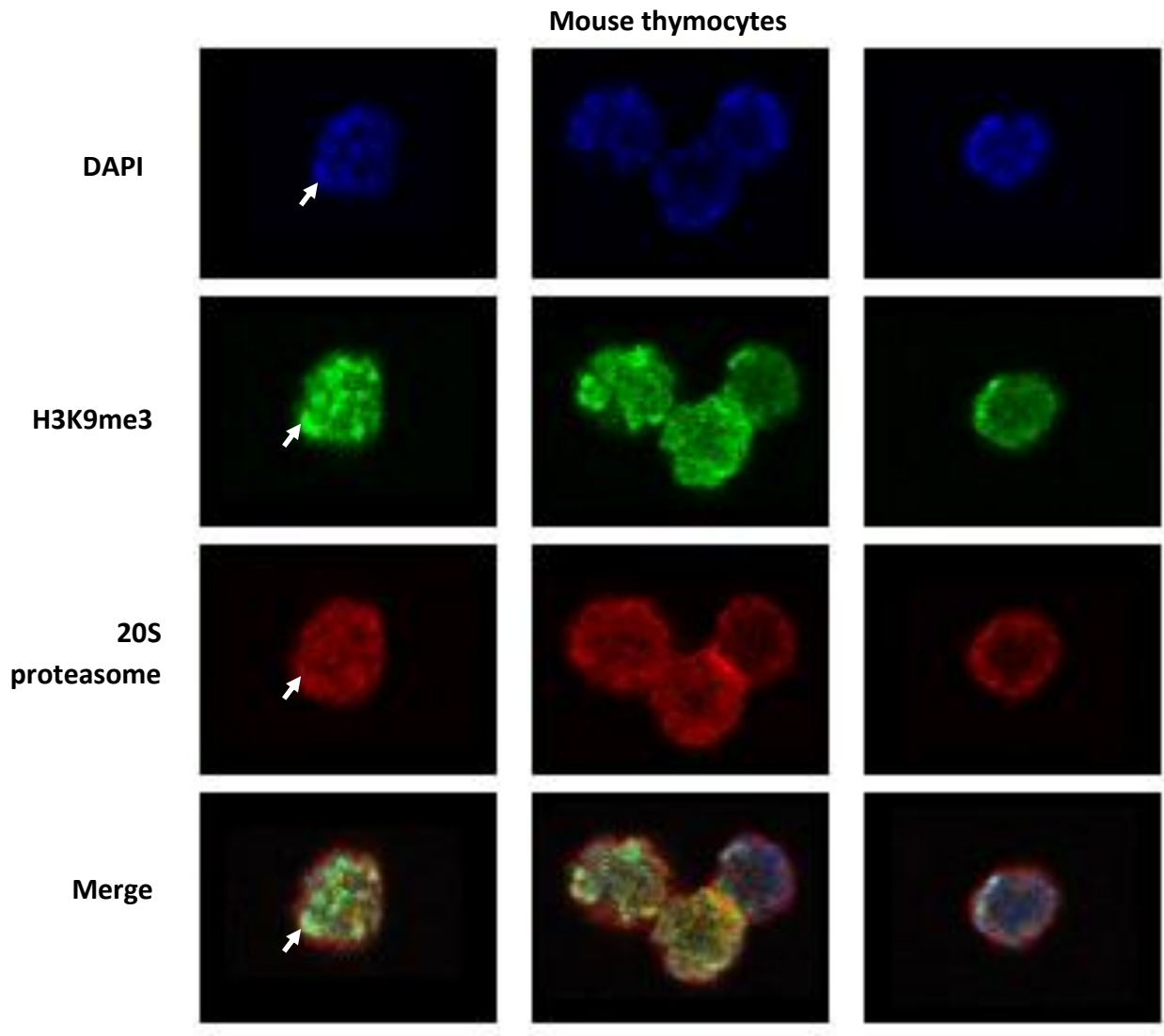


Figure 3.3 – Immunofluorescence staining against H3K9me3 and the 20S proteasome on mouse thymocytes. Thymocytes were obtained from ~4 weeks old male mice by teasing the thymus tissue and cell filtering. Cells were fixed and permeabilised with methanol and antibodies were pre-conjugated with Alexa Fluor® 488 and 594. H3K9me3 signal [green] overlaps significantly with the heterochromatic dense spots as shown on DAPI staining (blue). Notably, 20S proteasome moderately correlates with H3K9me3 signal, particularly at heterochromatic spots.

3.2.3 Proteasome inhibition upregulates heterochromatic transcription

So far, the results presented in this chapter suggested a link between heterochromatin and the proteasome both in the EBV-transformed human lymphoblastoid cell line and mouse thymus. According to the original hypothesis based on the finding on the heterochromatinised *FXN* gene in Friedreich's ataxia, it was thought that the proteasome might degrade RNAPII and thereby reduce the chances of successful transcription in heterochromatin. Clearly, one way of testing this hypothesis would be to inhibit the proteasome and observe its effect on transcription levels. So, both GM14926 cells and mouse thymocytes were cultured for 8 hours in the presence of DMSO or the classical proteasome inhibitor MG132 (final concentration 10 μ M) that had been dissolved in DMSO. RNA was extracted followed by cDNA synthesis and the transcripts levels of the repeats and genes indicated in figure 3.1 and 3.2 were analysed by Q-RT-PCR using the same primers as in the previous experiments. Because cDNA was synthesised with random hexamers and the primer sequences for genes are exonic, the results are attributed to a total transcript level which includes both primary transcript and mature messenger RNA.

As shown in figure 3.4, proteasome inhibition upregulated the transcription of centromeric and pericentromeric repeats in both cell lines to the highest extent, based on fold changes. It is noteworthy that these regions are mostly silenced in differentiated cells, but a basal level of transcription was detected on rare occasions [see general introduction 1.8.3.2]. In addition to these, there was a moderate level of upregulation of transposons. Inactive genes with high levels of H3K9me3 also exhibited a moderate to high level of upregulation, whereas housekeeping genes only displayed no or little upregulation compared to the others. A very high level of upregulation of constitutive heterochromatin as a response to MG132 fits well with the hypothesis that the proteasome can degrade RNAPII and provide another dimension for heterochromatic gene silencing.

Figure 3.4

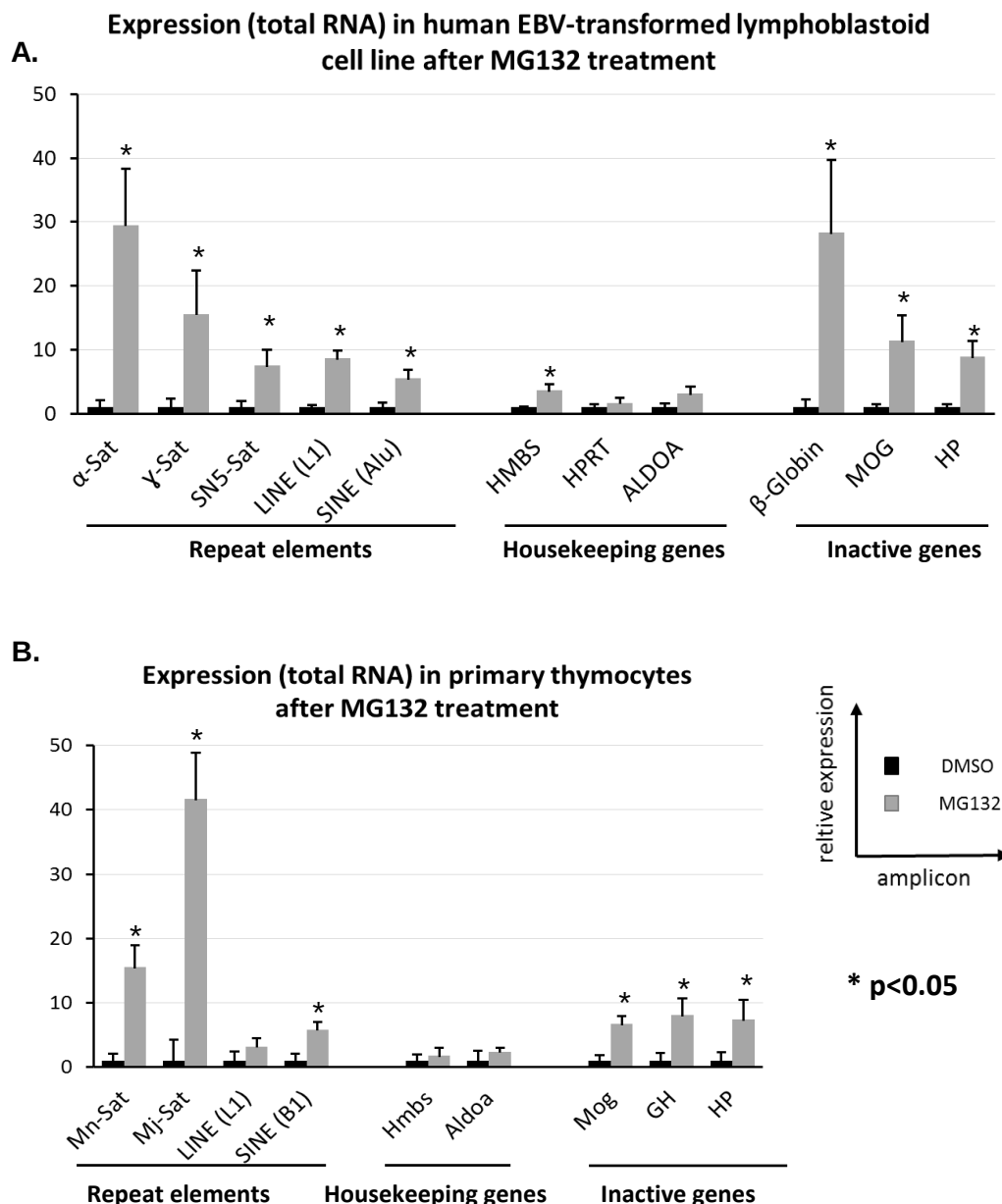


Figure 3.4 – Q-RT-PCR analysis of transcription levels in GM14926 cells and mouse thymocytes after MG132 proteasome inhibition. Treatment was performed for 8 hours and at a final concentration of 10 μ M. **[A] GM14926 cells.** Upregulation was highest in constitutive heterochromatin and lowest in housekeeping genes. **[B] Mouse thymocytes.** Consistent with GM14926, there was a marked upregulation in centromeric and pericentromeric repeats. [* p<0.05 student's t-test in comparison to the expression level obtained from the untreated sample. Data was normalised to β -actin expression. Error bars represent SEM of three different biological experiments, primers are exonic and cDNA was synthesised with random hexamers.]

3.2.4 Regression analyses showed a significant correlation between heterochromatic gene silencing and the proteasome

In order to determine the correlation between heterochromatin and the proteasome, linear regression analyses were performed using data presented in figures 3.1, 3.2 and 3.4. For this, average enrichment values for proteasome binding versus RNAPII or H3K9me3 levels were displayed as scatter plots using Microsoft Excel software. The correlation between enrichment patterns were visualised as a line of best-fit for which a Pearson's correlation coefficient (R^2) was calculated [Figure 3.5]. Clearly, there was a negative correlation between the RNAPII binding (X-axis) and the proteasome binding (Y-axis) both in human GM14926 cell line and mouse thymus [A]. In contrast, H3K9me3 levels correlated positively with the proteasome binding [B]. The same type of analysis was also performed for the response to MG132 treatment at total RNA level. Although the correlation level for the latter was high for the experiment with mouse thymus, a weak correlation was found in the EBV-transformed human cell line. This was thought to be because of the experimental variability for α -satellites and the β -globin gene [Figure 3.4A]. However, if these two outliers are taken out from the scatter plot, one can clearly see the correlation [represented with the green line.] This revealed that indeed there was a positive correlation between the upregulation response to proteasome inhibition and proteasome binding. Overall, regression analyses support the original hypothesis of RNAPII degradation by the proteasome at heterochromatic regions. This mechanism seems to be conserved in mammals as both human and mouse cells exhibited the same phenomenon.

Figure 3.5

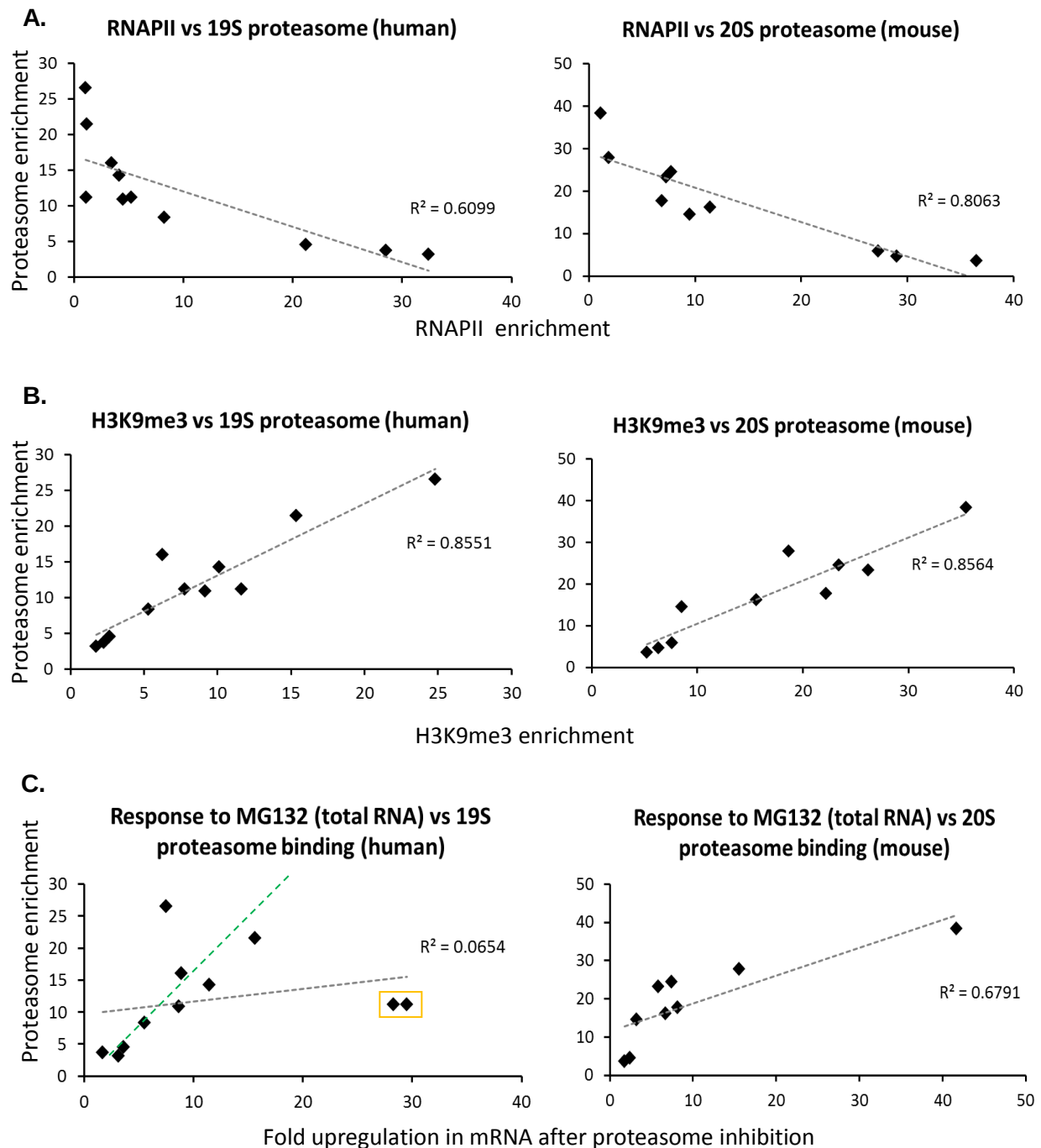


Figure 3.5- Regression analyses of proteasome ChIPs and post-proteasome inhibition expression data. Enrichment values of proteasome binding versus other factors were represented on scatter plots. Pearson's correlation coefficient R^2 values were also presented on plots. **[A] RNAPII versus proteasome.** There is a negative correlation between the two factors. **[B] H3K9me3 and proteasome.** There is a positive correlation. **[C] Upregulation by fold change versus proteasome binding.** Response to MG132 treatment is proportional with the proteasome enrichment levels. Green line indicates the best-fit after taking out the outliers in the yellow box. [Each dot represents average values for each primer from the figures 3.1, 3.2 and 3.4.]

3.2.5 Proteasome inhibition relieves PEV-silencing of the hCD2 transgene in mouse thymus

Position effect variegation (PEV) has been an invaluable tool for identifying factors that contribute to heterochromatic gene silencing and led to the identification of many chromatin modifiers including HP1 [see general introduction 1.5.1]. PEV effects are also known to be inherited by daughter cells, with implications for understanding epigenetic mechanisms. In mammals, PEV has been studied using hCD2 transgenic mice, which express the human CD2 surface marker specifically in mouse thymus and mature T cells [see general introduction 1.5.6]. Variegation patterns of hCD2 have been formerly shown to be sensitive to the dosage of heterochromatic factors, including H3K9me3, HP1 or histone acetylation (Festenstein *et al.*, 1999; Hiragami-Hamada *et al.*, 2009). It was therefore decided to see the effects of proteasome inhibition on PEV-silencing of the hCD2 transgene in transgenic mice (CBA.ca). For this purpose, mice expressing the hCD2 transgene (4 weeks old male littermates) were injected with the proteasome inhibitor PS341 which was developed for *in vivo* applications [see general introduction 1.7.2]. Mice were injected intraperitoneally for four consecutive days with a low dose [0.075 mg/kg], which was previously shown not to cause thymus atrophy (Maseda *et al.*, 2008). Thymus tissue was collected on the fifth day. This was because the bulk of thymocytes are known to be completely turned over every 5-7 days in young adult mice (Scolley *et al.*, 1980). Because PEV-silencing is typically established early in cellular life cycle, it was thought that the effect of proteasome inhibition on PEV could therefore be observed within this time frame. After the dissection, thymus tissue was teased in phosphate buffered saline and the cellularity of the thymus was assessed. No significant difference was found in cell numbers between the PS341 and physiological saline injected groups. Thymocytes were then fixed with 1% formaldehyde and then stained with antibodies against CD4 and CD8 surface glycoproteins as well as hCD2. A uniform population of cells which are double positive in CD4 and CD8 expression were selected [see supplementary figure S.10] and hCD2 expression was analysed in this cell population (Rothenberg and Taghon, 2005). Results showed that PS341 treated mice exhibited a lower degree of PEV silencing on hCD2 transgene ($p < 0.05$, Student's t-test.) [Figure 3.6]. Notably, there was no difference in the mean fluorescence intensities of hCD2 expressing cells. Thus, the observed difference was attributed to the PEV gene silencing effects rather than an increase in protein stability. Overall, this result supports the hypothesis that the proteasome provides an extra dimension for heterochromatic gene silencing. Because of epigenetic nature of PEV [see general introduction 1.5.1], it could also be speculated that the proteasome is involved in epigenetic inheritance of heterochromatic silencing.

Figure 3.6

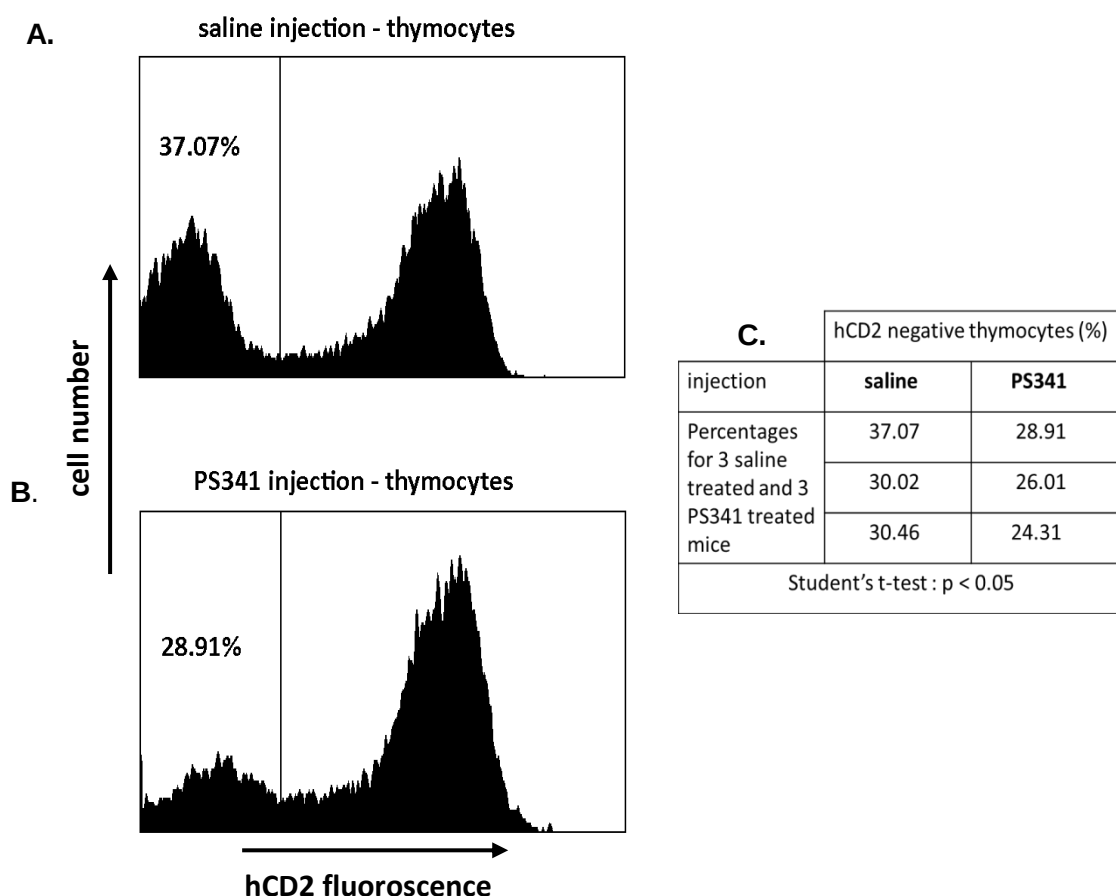


Figure 3.6 – Flow cytometry analysis of hCD2 expression in mice upon treatment with the proteasome inhibitor PS341. Transgenic mice were injected with physiological saline (control) or PS341 (0.075 mg/kg in saline solution) for four consecutive days. Thymus tissue was collected on the fifth day of treatment, teased apart and filtered in PBS to obtain thymocytes. CD4 and CD8 double positive thymocytes were selected to analyse hCD2 expression [see supplementary figure S.10]. **[A] hCD2 variegation in saline injected mice. [B] hCD2 expression of PS341 treated mice. [C] The percentage of hCD2 negative thymocytes.** PS341 treated mice exhibited significantly less PEV-silencing of the hCD2 transgene. Mean fluorescence intensity levels remained in the same range. [Each experiment refers to a different mouse.]

3.3 Discussion

Mammalian cells have evolved some chromatin-related mechanisms in order to silence improper gene expression or to avoid a high transcriptional noise that could arise from repetitive DNA. These could also have functional implications on nuclear architecture. Importantly, establishment of constitutive heterochromatin around centromeres is essential for kinetochore formation and efficient segregation of chromosomes during cell division (Guenatri *et al.*, 2004; Maison *et al.*, 2002; review-Peters *et al.*, 2008). It is a generally accepted phenomenon that the highly condensed structure of heterochromatin can inhibit transcription by restricting the access of essential factors as well as RNAPII [see general introduction 1.2.2]. Still, a basal level of transcription was reported for the highly heterochromatic centromeric or pericentromeric repeats, whose over-expression was linked to cancer [see general introduction 1.8.3.2] (review-Eymery *et al.*, 2009; review-Fisher and Merckenschlager, 2002; Martens *et al.*, 2005; Ting *et al.*, 2011). This suggests that low levels of RNA polymerase can somehow manage to reach DNA and transcribe it even in the case of a densely packaged chromatin structure. One way of reducing the chances of such undesired transcription could be directing heterochromatin into a peripheral nuclear position, where the rate of interaction with transcription machineries is much lower (Alcobia *et al.*, 2000; Andrulis *et al.*, 1998; Dietzel *et al.*, 2004; Hiragami-Hamada *et al.*, 2009). However, it is likely that mammalian cells could also have developed additional mechanisms in order to reduce the chances of RNAPII to reach the DNA and transcribe. Stemming from our original finding of a role for the proteasome in silencing the *FXN* gene in FRDA, evidence presented in this chapter suggested that a similar mechanism involving the proteasome might operate more widely to degrade RNAPII on heterochromatic DNA and thereby facilitate transcriptional silencing.

So far, many studies focusing on transcription-related functions of the proteasome were performed in *S. cerevisiae*. These reports emphasised a positive role for the proteasome on yeast transcription, primarily by the 19S regulatory particle which is thought to promote a remodeling action on the transcriptionally active chromatin [see introduction 3.1.1]. Whether such an effect is evolutionarily conserved in mammals, has remained unclear although some *in vitro* studies with human cell extracts were favouring this phenomenon (Fatyol and Grummt, 2008; Ferdous *et al.*, 2002). However, ChIP results in human cells and mouse thymus presented in this chapter show significantly less binding of both 19S and 20S proteasome particles on active housekeeping genes compared to inactive genes [Figure 3.1 and 3.2]. Although low levels of 19S proteasome may still have an advantageous effect on the progress of on-going transcription in mammals, this might not be linked to the proteolytic

roles of its functional partner 20S, whose inhibition caused no effect or slight upregulation of active genes [Figure 3.4]. Interestingly, heterochromatin enriched repetitive DNA and inactive genes were upregulated upon proteasome inhibition. Considering this and the positive correlation between H3K9me3 and proteasome binding [Figure 3.5], there appears to be a synergistic link between heterochromatin-mediated gene silencing and the proteasome in mammals. This was further supported by the fact that PEV silencing of the hCD2 transgene in mouse thymus is relieved upon inhibition of the proteasome. Immunofluorescence (IF) staining also showed co-localisation of H3K9me3 with the catalytic 20S proteasome subunit.

At this point, it is of special importance to note that the budding yeast *S. cerevisiae*, where most of the proteasome studies were performed, has heterochromatin only in a very restricted context mainly at telomeres and the ribosomal DNA gene arrays (review-Bannister and Kouzarides, 2011; review-Beisel and Paro, 2011; review-Kouzarides, 2007a; review-Xu and Zhu, 2010). Although the fission yeast *S. pombe* is known to have a higher order of heterochromatin structure and share some key features with mammals (e.g. HP1 and the H3K9 methylase) it is still restricted to a few regions in the genome. This is due, in large part, to the different nuclear architecture and paucity of repetitive DNA sequences in the yeast genome (review-Rando and Chang, 2009). Importantly, RNA interference was shown to be a strong inducer of heterochromatinisation and gene silencing in *S. Pombe*, whereas the presence of such a silencing pathway in mammalian cells has been under constant debate due to the lack of substantial evidence [see general introduction 1.4.2]. One possibility is that perhaps mammals have evolved other mechanisms to boost heterochromatic gene silencing. And it seems likely that the newly described role of the proteasome could be one of them.

Consistent with the findings presented here, a previous study on human cells reported that 20S proteasome inhibition elevated the levels of RNAPII and the active chromatin mark H3K4me3 in glucocorticoid responsive gene promoters, where significant binding of proteasome was detected (Kinyamu and Archer, 2007). Interestingly, another study reported that proteasome inhibition caused global histone hyperacetylation and hypomethylation (particularly at H3K9 and H3K27 residues) in the liver of rats treated with the proteasome inhibitor (PS341) (Oliva *et al.*, 2009). Moreover, a study with mouse embryonic stem cells reported that the proteasome degrades the RNAPII pre-initiation complex at cryptic gene promoters in the absence of an activator (Szutorisz *et al.*, 2006). Finally, another interesting example comes from the HIV promoter, where transcription is repressed by the proteolytic activities of the 26S proteasome during the viral latency period

(Lassot *et al.*, 2007). Although previous studies pointed out a role for the proteasome in mediating mammalian gene silencing, they did not specifically take chromatin status into account. In this study, genes representing different chromatin status showed a negative correlation between RNAPII levels and proteasome binding [Figure 3.5], suggesting that proteasome mediated silencing mechanisms could happen in a broader context. In order to address this more systematically, genome-wide ChIP sequencing will be performed. In the previous chapter, restoration of RNAPII levels at the heterochromatin-silenced *FXN* locus upon proteasome inhibition was observed in the presence of alpha-amanitin. The purpose of inhibiting transcription was to ensure that no new RNAPII can replace the degraded RNAPII over time. Therefore, it would be interesting to check the levels of RNAPII at other heterochromatic locations upon proteasome inhibition, in the presence and absence of transcription blockers.

What is the mechanistic link between the proteasome and heterochromatin? There is no study yet which directly addressed this issue. Interestingly, a recent proteomics study reported that HP1 γ is co-immunoprecipitated with the 11S proteasome particle in human cells (Vermeulen *et al.*, 2010). Therefore, it would be worth to repeat the ChIPs presented in this study with the 11S proteasome. As mentioned in the previous chapter, HP1 γ was also shown to regulate the silencing of the latent-phase HIV promoter, where transcription was also found to be repressed by the 20S proteasome (du Chene *et al.*, 2007; Lassot *et al.*, 2007). Moreover, results presented in the previous chapter also showed enriched HP1 γ binding on the silenced *FXN* gene at the site where RNAPII is degraded by the proteasome [Figure 2.3 and 2.14]. Therefore, it would be interesting to investigate the link between HP1 γ and the proteasome further, possibly by knocking down HP1 γ and testing its effect on proteasome binding via ChIP. The tight correlation between H3K9me3 and the proteasome also makes it possible that H3K9me3 mark by itself can somehow recruit the proteasome. It would therefore be interesting for example to knockdown or inhibit the H3K9 specific methyltransferase SUV39H by chemical inhibitors (Illner *et al.*, 2010) and assess the effect on the binding of the proteasome. Moreover, proteasome immunoprecipitation on chromatin extracts combined with mass spectrometry analysis would allow the identification of other proteasome associated factors that might be important in its recruitment to heterochromatin. The proteasome is known to recognise its targets mostly by ubiquitination. However, 11S proteasome regulators are known to direct a low level of proteasome activity in a ubiquitin-independent context, although how they recognise the substrates remain unclear [see general introduction 1.7.1]. Therefore it is also possible that RNAPII degradation in heterochromatin could be independent of ubiquitination.

3.4 Contributions

Cihangir Yandim is responsible for the ideas and experiments presented in this chapter. Theona Natisvili; an MSc student supervised by Cihangir Yandim, provided on-bench help for some ChIP experiments.

CHAPTER 4

HETEROCHROMATIN EFFECTS IN SEXUAL DIMORPHISM

4.1 Introduction

4.1.1 What is sexual dimorphism?

Phenotypic and behavioural differences are seen in all animal species. In humans, such sexual dimorphisms are known to cause different predispositions to disease as well as their response to treatment. Classical examples include cardiovascular disease, which is prevalent more in men during adulthood with a higher rate of occurrence in post-menopausal women; asthma, which is more common in boys than girls following puberty; some autoimmune diseases (e.g. Lupus, rheumatoid arthritis), which are more prevalent in women throughout life; several psychiatric disorders (e.g. major depression more common in women); neurodegenerative diseases (e.g. Parkinson more common in men but Alzheimer's in women) and finally cancer (e.g. colorectal cancer and hepatocellular carcinoma more prevalent in men, thyroid cancer more common in women) (review-Kaminsky *et al.*, 2006; review-Ober *et al.*, 2008). Also, gender differences in basal and lipid metabolisms are well described (review-Wang *et al.*, 2011b). Moreover, females are known to have increased T-cell numbers and immune response (review-Bouman *et al.*, 2005). Finally, another interesting difference is gender specific brain anatomies (review-Paus, 2010).

The sexual dimorphism phenomenon explained above could be either an indirect effect of sex hormones or a direct result of sex chromosome specific gene products. Microarrays in mice revealed thousands of autosomal genes that are differentially expressed (~1.2 fold, for most of them) between genders, in brain (14%) and liver (70%) (Yang *et al.*, 2006). Furthermore, many genes that are not subject to dimorphic expression patterns exhibit various levels of splicing isoforms in mammals (Blekhman *et al.*, 2010; Su *et al.*, 2008). In line with this, several differences were observed in the components of the *Drosophila* splicing machinery (review-Lalli *et al.*, 2003; Telonis-Scott *et al.*, 2009). Another interesting finding in mouse is the identification of single nucleotide polymorphisms (SNP) which were linked to the gender specific variation in gene expression, implying that some *trans*-acting factors recognise specific sequence elements as *cis*-regulatory regions in one sex but not in the other (Bhasin *et al.*, 2008). In addition, there is growing evidence suggesting that epigenetic mechanisms could be sexually dimorphic. Interestingly, expressions of hundreds of mouse genes were shown to be affected by paternal diet via a yet-unclarified mechanism that involves DNA methylation (Carone *et al.*, 2010).

4.1.2 Hormonal effects

Sex hormones (i.e. estrogen and testosterone) can influence a large number of genes and also affect the production rate and response to other hormones (i.e. thyroid, progesterone, androgen..etc) (review-Ober *et al.*, 2008). For instance, the regulation of body fat distribution is influenced by changes in gonadal hormones, including the adipose-derived hormone leptin (Pasquali, 2006). Also, a difference in steroid hormone levels is known to cause sexual dimorphisms in liver metabolism (review-Roy and Chatterjee, 1983). Importantly, these differences are lost in the liver of gonadectomised male mice (van Nas *et al.*, 2009). Many more examples can be given regarding to hormonal influence on sexual dimorphism. In general, they share mechanisms where hormone receptors act by forming complexes with transcription factors or co-activator complexes (e.g. estrogen and progesterone receptor associate with transcription factors to form the SMRT complex) or directly with components of epigenetic machinery (e.g. androgen receptor associates with the histone demethylase LSD1) (review-Kaminsky *et al.*, 2006; review-McCarthy *et al.*, 2009).

4.1.3 Sex chromosome complement effects

Sex chromosome complement is defined as the composition of sex chromosomes contained within each cell (i.e. XY in normal males, and XX in normal females). Sexual dimorphisms could also be triggered directly by sex chromosome complement independently of hormonal effects. First lines of evidence regarding to this came from the observation that male and female pregonadal mice embryos were influenced by X or Y chromosomes (Burgoyne, 1993; Burgoyne *et al.*, 1995; Thornhill and Burgoyne, 1993). In line with this, expressions of hundreds to thousands of genes were reported to be sexually dimorphic in mouse and bovine blastocysts before gonadal differentiation takes place (Bermejo-Alvarez *et al.*, 2010; Kobayashi *et al.*, 2006). These differences in gene expression may be due to the effects of sex chromosomes on gene regulation mechanisms. Also, differences in DNA methylation levels were reported to be dependent on sex chromosome complement in mouse embryonic stem cells and induced pluripotent stem cells (Durcova-Hills *et al.*, 2004; Durcova-Hills *et al.*, 2006; Maherali *et al.*, 2007; Ooi *et al.*, 2010; Zvetkova *et al.*, 2005). Although the underlying mechanisms remain unclear, these studies suggest a significant role of sex chromosome complement in determining sexual dimorphisms. In order to have a better insight into these sex chromosome complement effects, one needs to understand the nature of sex chromosomes.

4.1.4 X chromosome and its inactivation

In normal conditions where females carry two X chromosomes and males carry one X and one Y chromosome, compensatory mechanisms have evolved in order to deal with a potential gender imbalance based on the presence of one extra X chromosome in females. Importantly, abnormalities in the number of X chromosomes can give rise to Turner's syndrome (XO) or Klinefelter's syndrome (XXY) in humans (review-Lanfranco *et al.*, 2004; review-Sybert and McCauley, 2004). Various X compensation mechanisms were reported among diverse animal species in order to ensure equivalent gene expression in males and females. One example can be given from *Drosophila*, where the expression of X chromosome genes in males is doubled by the action of two male specific non-coding RNAs (*roX1* and *roX2*) and MOF dependent H4K16 acetylation (review-Taipale and Akhtar, 2005).

In mammals, compensation is achieved by the inactivation of one random X chromosome (XCI: X-chromosome inactivation) early during development [e.g. day 5.5 in mouse embryogenesis] (Lyon, 1961; Sugimoto and Abe, 2007). Briefly, this process involves the expression of the X inactive specific transcript, *Xist*, that is thought to coat the inactivated X chromosome via interactions with conserved DNA motifs and the scaffold protein, SAF-1 (Fackelmayer, 2005; Penny *et al.*, 1996; review-Wutz, 2003). Evidence now suggests that *Xist* may function together with LINE elements, which are thought to direct genes into a 'silenced compartment' within the nucleus (Bailey *et al.*, 2000; Chow *et al.*, 2010). A repressive compartment is established by the *Xist* mediated recruitment of polycomb complexes PRC1 and PRC2 (Leeb *et al.*, 2010; Schoeftner *et al.*, 2006). Therefore, H3K27 methylation and H2AK119ub are predominant marks within the inactivated X (Xi). In addition, other histone modifications were detected at the Xi; possibly as a secondary effect. These include H3/H4 hypoacetylation, H3K9 hypermethylation (mainly H3K9me2 but also H3K9me3) and H3K4 hypomethylation (Brinkman *et al.*, 2006; Rougeulle *et al.*, 2004; Valley *et al.*, 2006). Stabilisation of the repressive compartment seems to be mediated by DNA hypermethylation (by the DNA methyltransferase DNMT1) on the inactive gene promoters (Cotton *et al.*, 2011; Sado *et al.*, 2000). Other proteins associated with the Xi include histone variant macro H2A (Costanzi and Pehrson, 1998), AT rich binding transcriptional repressor SATB1 (Agrelo *et al.*, 2009), trithorax group HKMT ASH2L (Pullirsch *et al.*, 2010) as well as the hinge domain DNA binding and stabilising protein SMCHD1 (Blewitt *et al.*, 2008). Importantly, certain genes on Xi escape the silencing process and these are thought to be involved in key events during sexual development and dimorphisms (review-Arnold, 2011; Carrel and Willard, 2005). Mechanistically, escape from XCI has not been clearly defined yet; however, genomic environment, surrounding boundary elements (e.g. CTCF binding

sites) and possibly specific distribution patterns of LINEs were implicated (Filippova *et al.*, 2005; Popova *et al.*, 2006; review-Wutz, 2011)

4.1.5 Y chromosome and male gender development by *SRY*

Some of the XCI escapees have homologues on the Y chromosome (e.g. JARID1c and JARID1d; see discussion 4.3.3 for further explanation), whereas some others do not have counterparts. One example for the latter is Y specific *SRY* (Sex determining region of Y), which is the primary gene that is involved in the gonadal development of males (review-Goodfellow and Lovell-Badge, 1993; Koopman *et al.*, 1991). *SRY* encodes a ~200 aminoacid conserved protein that has a DNA binding domain [which recognises the motif: (A/T)ACAA(T/A)] known as the “high mobility group (HMG)” box (Ferrari *et al.*, 1992). Characterisation of the HMG box led to the identification of more than 20 HMG-box containing proteins that show homology with *SRY*; members of the SOX (*SRY*-like box containing) family. *SRY* and SOX proteins can interact with the minor groove of DNA forcing the helix apart to create a 90° bend and primarily exert transcription activating functions (review-Lefebvre *et al.*, 2007).

SOX family proteins are known to contribute to a number of cellular differentiation processes (e.g. neurogenesis, lymphogenesis, etc.) acting both as transcriptional activators or repressors depending on the context (review-Lefebvre *et al.*, 2007). Among SOX family members, SOX9 shows relatively higher homology with *SRY* and is of particular importance during male development (review-Sekido and Lovell-Badge, 2009). Interestingly, *SRY* and SOX proteins are able to transcriptionally regulate each other or in some cases provide positive feedback to themselves (e.g. SOX9). Early in the formation of the genital ridge, SOX9 is expressed in both sexes and its activity is hampered by the accumulated β -catenin as a response to RSPO1/WNT4 signalling that takes place at subsequent stages (Chang *et al.*, 2008; Liu *et al.*, 2009a; Maatouk *et al.*, 2008). However, expression of *SRY* together with the transcription factor SF1 (also known as “ZFM1”) boosts SOX9 expression in male genital ridge (Hiramatsu *et al.*, 2009; Kidokoro *et al.*, 2005; Sekido and Lovell-Badge, 2008). Very little is known about how the expression of *SRY* is regulated in the first place. Still, insulin signalling and SF1 expression as well as transcription factors CBX2 and WT1 were suggested to regulate the transcription of *SRY* (Nef *et al.*, 2003; Pilon *et al.*, 2003; review-Sekido and Lovell-Badge, 2009). Finally, upregulated SOX9 promotes the testis pathway by activating the expression of the Anti-Mullerian hormone (AMH) and triggers sertoli cell differentiation (Kent *et al.*, 1996; Morinaga *et al.*, 2007).

4.1.6 Four core genotype mouse model

Development of “four core genotype” mice model, where the male sex determining Sry gene is deleted from the Y chromosome and introduced into an autosome, allowed scientists to distinguish sex chromosome complement effects from hormonal influences by reversing the phenotypic sex of the animal (De Vries *et al.*, 2002a). Accordingly, crossing XY⁻sry male with a normal female (XX) results in progeny with two types of male (XXsry and XY⁻sry) and two types of female (XX and XY⁻) that differ in sex chromosome complement. Both XXsry and XY⁻sry mice were reported to have similar testosterone levels in prenatal and neonatal stages (De Vries *et al.*, 2002a; Gatewood *et al.*, 2006; Palaszynski *et al.*, 2005; Wagner *et al.*, 2004). Using this model system, several behavioural traits and anatomical differences in various tissues including brain were reported to be solely dependent on X or Y chromosome but not gender (review-Arnold and Chen, 2009). Moreover, a ‘ying-yang’ effect was elucidated in autoantigen-specific immune response, where proliferative response and cytokine production was shown to be stimulated by the presence of the Y chromosome and conversely inhibited by the male sex determining factor Sry (Palaszynski *et al.*, 2005).

4.1.7 Hypothesis

Sexual dimorphisms have long been thought to be caused only by hormonal differences. Although more recent studies addressed possible differences in gene regulation mechanisms due to sex chromosome complement effects, the characteristics of such phenomena have not been elucidated yet. In this study, it was hypothesised that at least part of the sexual dimorphisms could be due to gender differences in heterochromatin mediated gene silencing mechanisms. Such possible effects may be a result of PEV- like variegation in endogenous gene expression and could be triggered by various components of heterochromatin that are affected by sex chromosomes.

4.2 Results

4.2.1 Sexual dimorphism in heterochromatic gene silencing is caused by X chromosome complement

Mechanisms in chromatin mediated gene regulation can provide solid explanations for various aspects of development and their dysregulation can contribute to numerous pathologies including cancer and neurodegenerative diseases. The strong potential of chromatin on determining the expression status of a given gene makes it also an essential candidate for explaining sexual dimorphisms, which are exemplified by the high number of mammalian gene expression differences (Carone *et al.*, 2010; Yang *et al.*, 2006). Indeed, epigenetic mechanisms involving DNA methylation have recently been suggested to give rise to such sexual dimorphisms in gene expression (Bhasin *et al.*, 2008; Carone *et al.*, 2010; Durcova-Hills *et al.*, 2004; Durcova-Hills *et al.*, 2006; Maherali *et al.*, 2007; Ooi *et al.*, 2010; Zvetkova *et al.*, 2005). However, most of these studies involve *in vitro* research and address early developmental events in mouse. Whether potential differences in chromatin mediated gene regulation takes place *in vivo* and remains during adulthood, needs to be investigated and characterised. In order to address this issue, this study took advantage of the archetypal epigenetic phenomenon of position effect variegation (PEV), which could be studied with the hCD2 transgenic system in mouse [see general introduction 1.5.1 and 1.5.6].

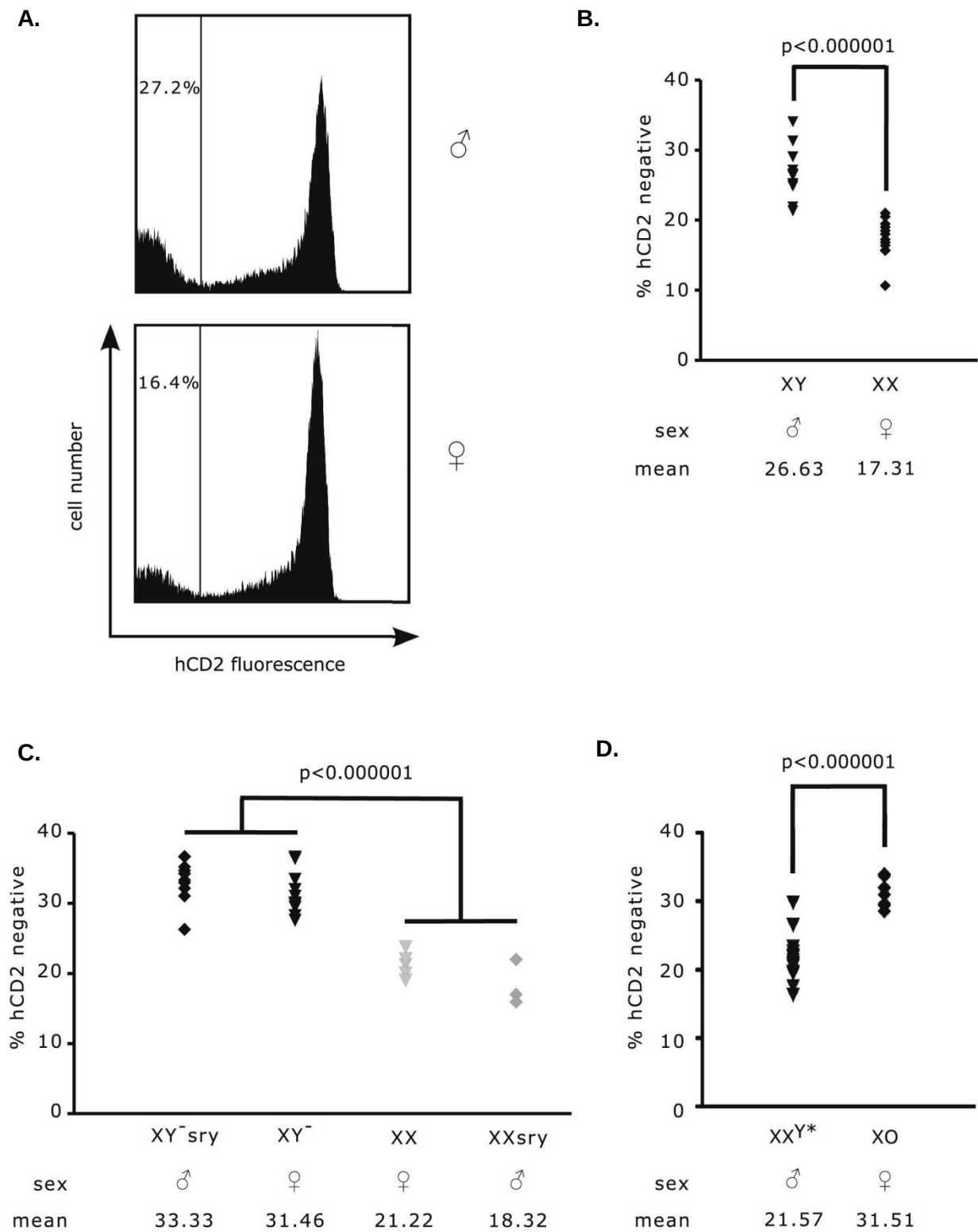
Because the expression of heterochromatin-adjacent hCD2 transgene exhibits sensitivity to chromatin modifiers such as HP1 (Festenstein *et al.*, 1999; Hiragami-Hamada *et al.*, 2009), it was postulated that any gender difference in the variegation patterns of hCD2 would imply potential variations in the abundance or function of chromatin factors in different sexes. Therefore, hCD2 levels in heterozygous transgenic mice (Ph.D. thesis-Festenstein, 1996; Festenstein *et al.*, 1996) were analysed by flow cytometry (FACS) using peripheral T lymphocytes and thymocytes from 6 – 8 week littermate mice [Figure 4.1A and 4.1B, thymus data not shown]. Mature T cells in peripheral blood could either exhibit the CD4 or CD8 surface marker (review-Rothenberg and Taghon, 2005). In order to be able to compare any differences in the PEV, it is essential to select a uniform population of cells. Therefore, at least 20,000 CD4 single positive cells in peripheral blood were selected in order to study the expression of the hCD2 transgene [see supplementary figure S.10]. Indeed, a marked difference was observed in the flow cytometric (FACS) analysis of hCD2 levels between males and females [Figure 4.1A and B]. Interestingly, the transgene was silenced in a larger proportion of cells in males compared to females and this observation was found to be

statistically significant ($p < 0.000001$) by using the one-way ANOVA test with generalised linear model using the NCSS statistical analysis software.

Based on previous reports which elucidate both hormones (review-Ober *et al.*, 2008; van Nas *et al.*, 2009) and sex chromosome complements (Bermejo-Alvarez *et al.*, 2010; Burgoyne, 1993; Kobayashi *et al.*, 2006; Ooi *et al.*, 2010) as potential drivers of sexual dimorphisms, it was crucial to ask whether the difference on heterochromatic hCD2 gene silencing was due to sex or genetic factors. In order to address this, hCD2 transgenic mice (females, XX, CBA.ca background) were crossed with four core genotype mice (males, XY^{sry}, C57BL/6 background) (De Vries *et al.*, 2002b). The progeny was similarly analysed by flow cytometry and the extent of hCD2 silencing was interestingly found to be higher in XY^{sry} females and XY^{sry} males than in XX females and XX^{sry} males ($p < 0.000001$), whereas sex differences had no significant effect in the two-way ANOVA analysis [Figure 4.1C]. Based on this, it was concluded that sex chromosome complement rather than sex determines the extent of heterochromatic hCD2 silencing, with two X chromosomes correlating with less silencing and the XY chromosome complement resulting in more silencing.

One obvious question to ask at this point was whether the presence of the Y chromosome is introducing a silencing effect or is it the absence of one extra X chromosome that contributes to the upregulation of the hCD2 transgene. To clarify this, hCD2 XX females were crossed with X^{Y*}O males (predominantly random bred MF1 albino background) where the Y chromosome is attached to one of the X chromosomes (Burgoyne *et al.*, 1998; Eicher *et al.*, 1991) to produce XX^{Y*} and XO females. The same hCD2 expression analysis on peripheral CD4⁺ T cells was performed on 6-8 weeks old littermates and it was found that XX^{Y*} males silenced the transgene significantly less ($p < 0.000001$, one-way ANOVA) than XO females [Figure 4.1D], which independently demonstrates an underlying role of sex chromosome complement rather than sex in heterochromatic hCD2 silencing. Importantly, the extra X chromosome was linked to a decreased silencing as the transgene was expressed more in the presence of the second X even when it was accompanied by a Y chromosome. Hence, it was concluded that the difference in X chromosome complement, rather than the presence or absence of the Y chromosome, determined the extent of hCD2 silencing. Bearing in mind that hCD2 transgene exhibits sensitivity to heterochromatin factors, one may expect that epigenetic chromatin mechanisms can be affected by the X chromosome complement but not gender and thereby contribute to sexual dimorphisms.

Figure 4.1



See next page for figure legend.

Figure 4.1 – FACS analysis to show sexual dimorphism in PEV-like heterochromatic gene silencing. [A] Histogram plots for the flow cytometry analysis of hCD2 expression. Analysis has been performed on at least 20,000 peripheral CD4⁺ T cells obtained from 6-8 week old heterozygous males (top panel) and females (bottom panel). The vertical line separates hCD2-negative population from the hCD2-positive population. hCD2 ‘silent’ cells are more prevalent in males (27.2%) than in females (16.4%). [B-D: Scatter plots and ANOVA analyses for hCD2 silencing. Each point represents the result obtained from a single transgenic mouse.] [B] **Comparison of hCD2 expression in transgenic males and females.** Higher silencing was observed in males. [C] **Comparison of hCD2 expression on four core genotype mice.** The proportion of hCD2-negative cells is lower in mice with two X chromosomes than in mice with an X and Y chromosome, irrespective of sex. [D] **Comparison of hCD2 expression levels on XX^{Y*} and XO backgrounds.** The proportion of hCD2-negative cells is lower in the presence of the two X chromosomes than in the presence of one X chromosome, even when attached with a Y chromosome.

4.2.2 Defining a set of autosomal genes influenced by sex chromosome complement

Having revealed the fact that the expression of heterochromatic hCD2 transgene is dependent on sex chromosome complement, it was wondered whether autosomal genes in adult mice are also affected by the same phenomenon. In order to address this, transcription profiling was performed on thymuses of four core genotype mice, sacrificing three individual animals per group. Tissue dissection was carried out on 6-8 week old littermates and RNA was extracted followed by cDNA and cRNA synthesis. Hybridisation was performed and data was analysed as explained in materials and methods 6.16. A previous large-scale mouse study reported that a high percentage of genes display less than 1.2 fold-changes between males and females (Yang *et al.*, 2006). Interestingly, the difference in the expression of the hCD2 transgene was of the same magnitude here [Figure 4.1A, the ratio of the percentage of hCD2-positive cells between XX and XY mice was $84/73 = 1.15$]. Therefore, genes that exhibit average fold-changes equal or larger than 1.2 with p-values less than 0.05 were considered to be differentially expressed [Student's t-test, (Shi *et al.*, 2008)].

Following this protocol, 1038 autosomal genes were shown to be differentially expressed in XY^{sry} and XX^{sry} males [Figure 4.2, black]. Because sex hormone levels were shown to be at the same level in these mice (De Vries *et al.*, 2002b; Gatewood *et al.*, 2006; Palaszynski *et al.*, 2005; Wagner *et al.*, 2004), differences in transcription profiles could be attributed to sex chromosome complements. Similarly, 1218 autosomal genes were revealed in the comparison of XX and XY⁻ females [Figure 4.2, white], 369 of which were affected by sex chromosome complement in both males and females (yellow). Experiments and analyses in the following sections will focus on these 369 'sex chromosome complement-sensitive (SCS)' genes. Expression differences of SCS genes in four core genotype mouse thymuses were validated with quantitative real time PCR using the same RNA that was processed for microarrays [see supplementary figure S.12]. A complete list of all SCS genes is also given on supplementary table S.1. All Affymetrix data as well as a metadata spreadsheet can be reached from NCBI Gene Expression Omnibus (Edgar *et al.*, 2002) with GEO accession number GSE21822 [<http://www.ncbi.nlm.nih.gov/geo/>] .

Figure 4.2

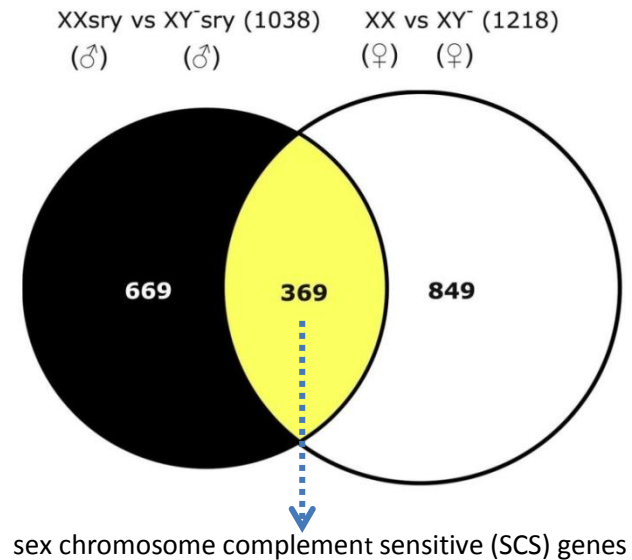


Figure 4.2 – A Venn diagram that represents differentially expressed genes in microarrays.

Transcription profiles were compared on XXsry vs XY^{sry} males on one hand (black), and XX vs XY⁻ females (white) on the other. Differentially expressed genes (>1.2-fold, Student's t-test $p < 0.05$) of both groups overlap on 369 genes (yellow), which are sensitive to sex chromosome complement. These were called “sex chromosome complement-sensitive (SCS) genes”. SCS genes provide a valuable set for further experiments on studying sex chromosome effects on autosomal gene regulation. [369 sex chromosome complement sensitive genes were listed on supplementary table S.1.]

4.2.3 Functional classification of sex chromosome complement-sensitive genes

In order to uncover the biological significance of SCS genes, a gene ontology and pathway analysis was performed using the online DAVID software with T cell transcriptome as background list [<http://david.abcc.ncifcrf.gov/> (Dennis *et al.*, 2003; Huang da *et al.*, 2009a, b)] . GO term analysis of over-represented genes [Fisher's exact test of over-representation $p < 0.05$; fold enrichment ≥ 1.5] revealed that approximately a quarter of SCS genes encode transcription related proteins including crucial transcription factors such as HMG box factor Sox4, zinc finger factor Sp1 and bromodomain proteins (Brd2, Brd4 etc.) as well as chromatin modifiers such as H3K4 specific histone methyltransferases Ash1l and Mll -3 and -5 [Figure 4.3A]. A significant proportion of SCS genes (~15%) was also found to be those encoding proteins involved in RNA processing machinery, including splicing factors (Fusip1, Sf3b1, Rbm4 etc.), RNA motif recognition proteins (Sltn, Taf15, Msi2 etc.) and transcription termination related factor Xrn1. Consistently, it is well known that splicing isoforms can contribute to sexual dimorphisms in mammals (Blekhman *et al.*, 2010; Su *et al.*, 2008) and components of the *Drosophila* splicing machinery comprises gender specific differences (review-Lalli *et al.*, 2003; Telonis-Scott *et al.*, 2009). Moreover, genes related to post-translational modifications of proteins (e.g. Cdk7, Csnk2a1, Map4k4 etc.) were also enriched among the SCS genes. Enrichment of transcription and chromatin related factors as well as RNA processing and protein modifying proteins is consistent with the hypothesis that some aspects of gene regulation mechanisms could be affected by sex chromosome complement. In addition to these, genes encoding products involved in lipid metabolism (Pten, Pigd, Pisd etc.) and other aspects of cellular metabolism such as metal ion binding (Zkscan3, Atr, Mex3c etc.) were also detected within the SCS gene set. This could be a reflection of already described sexual dimorphisms in basal and lipid metabolism (review-Mittendorfer, 2005). The rest of the genes seem to have function in relation to the cytoskeleton (Mtss1, Cald1, Arpc2, etc.) and focal adhesion (Tns1, Tln1, Arpc2 etc.).

Interestingly, KEGG pathway analysis [Figure 4.3B] revealed that SCS genes comprise components of focal adhesion pathway, consistent with the fact that males generally have stronger skin, muscle and skeletons (review-Ober *et al.*, 2008). Sexual dimorphisms in insulin resistance and diabetes are also well defined (Jaworski *et al.*, 2011; Macotela *et al.*, 2009). In line with this, components of the insulin signalling pathway also seem to be affected by the sex chromosome complement. Furthermore, pathways involved in T cell biology (Jak-STAT and mTOR) and components of natural killer cell mediated cytotoxicity were represented and these could be explained by the fact that there are indeed immune system differences between genders (review-Bouman *et al.*, 2005; review-Kaminsky *et al.*,

2006; Palaszynski *et al.*, 2005). Finally, pathways in relation to prostate, endometrial, non-small lung cancers as well as acute myeloid leukemia were also pronounced among SCS genes. This is also consistent with previous reports addressing gender differences in the predisposition and progression of these cancers (review-Ober *et al.*, 2008). Although many hormone receptor pathways (including, sex and thyroid hormones) were revealed in the pathway analysis of mice with different genders [data not shown], these seem to be eliminated in the SCS gene set analysis where only mice with the same sex were compared by only taking sex chromosome complement into account.

Figure 4.3

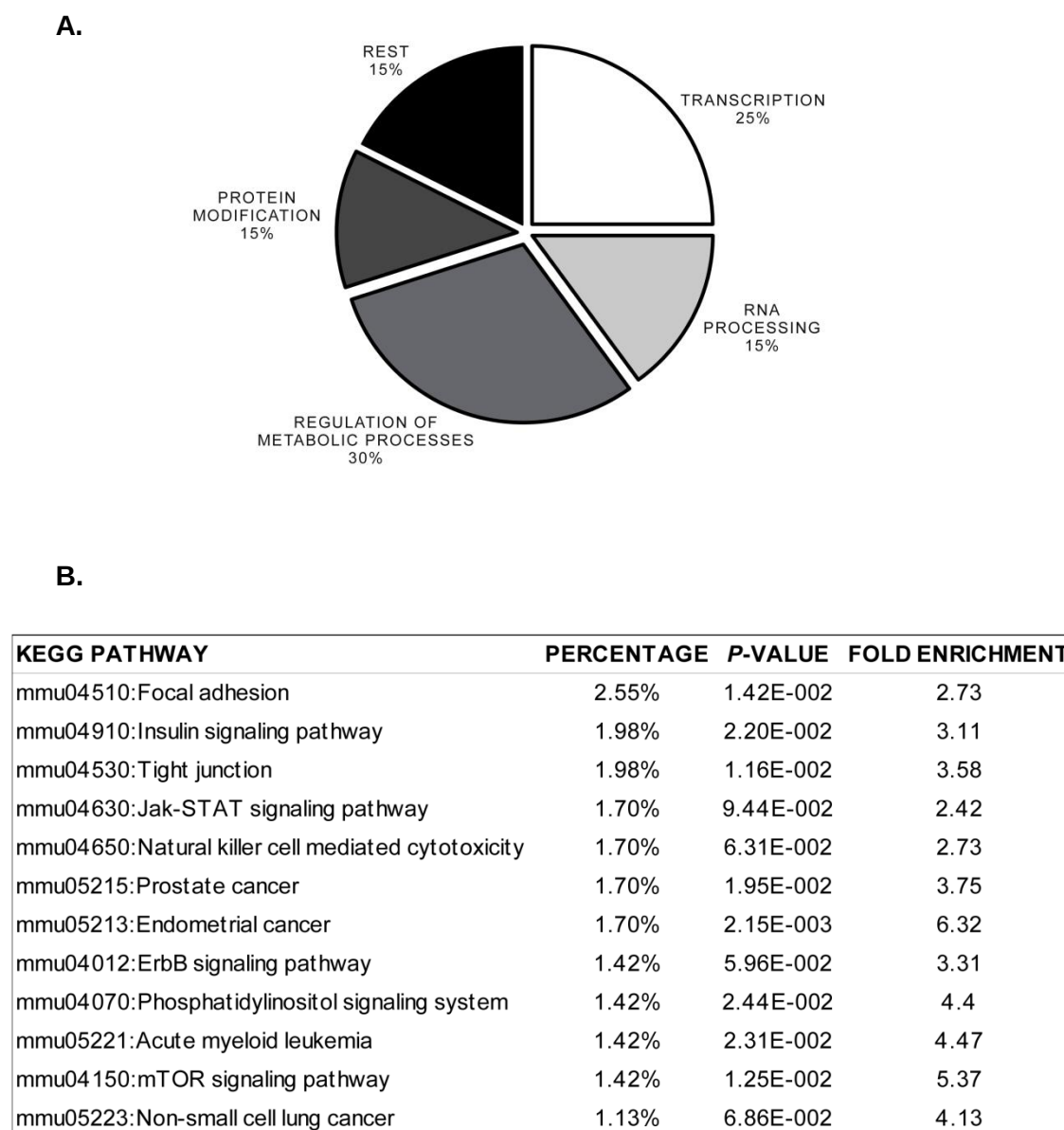


Figure 4.3 - Functional classification of sex chromosome complement-sensitive autosomal genes. [A] **Pie chart of GO term analysis.** The breakdown of genes is given according to main functional categories following GO analysis by using DAVID. [B] **Summary of KEGG pathway analysis.** Enriched KEGG pathways obtained after analysis of the sex chromosome-sensitive genes using DAVID.

4.2.4 Analysing the direction of sex chromosome complement effects

Expression of the heterochromatic hCD2 gene was shown to be affected by the sex chromosome complement [Figure 4.1] and it seemed to be positively affected by the presence of the second X chromosome rather than gender. Therefore, it was wondered whether the direction of sex chromosome complement effect on autosomal SCS genes is consistent with the hCD2 study or not. In order to address this, all genes represented in Affymetrix arrays (probe set level) were analysed using density plots drawn by the R Bioconductor. Average log₂ expression values were compared by importing microarray values from XX and XY backgrounds to the X and Y – axes of the density plots respectively. These plots revealed that most of the SCS genes (red) were indeed expressed at higher levels in XX^{sry} males than in XY^{sry} males [Figure 4.4A] which mirrors the result for the hCD2 transgene. Considering the fact that both males have the same testosterone levels, this effect was linked to sex chromosome complement in agreement with the hCD2 experiments described above. On the contrary though, an opposite direction of sex chromosome complement effect was found in the comparison of XX and XY females [Figure 4.4B]. This could be explained by a repressive effect due to an extra X chromosome in females and/or an activating effect raised by the presence of one Y chromosome in a *Sry* negative background. Apparently, this gene set responds to the sex chromosome regulatory inputs; however, the direction seems to be dependent on gender unlike the hCD2 reporter silencing phenomena. At this point, it could be speculated that sex hormones possibly provide an additional regulatory layer for the sex chromosome complement sensitivity on autosomes.

Figure 4.4

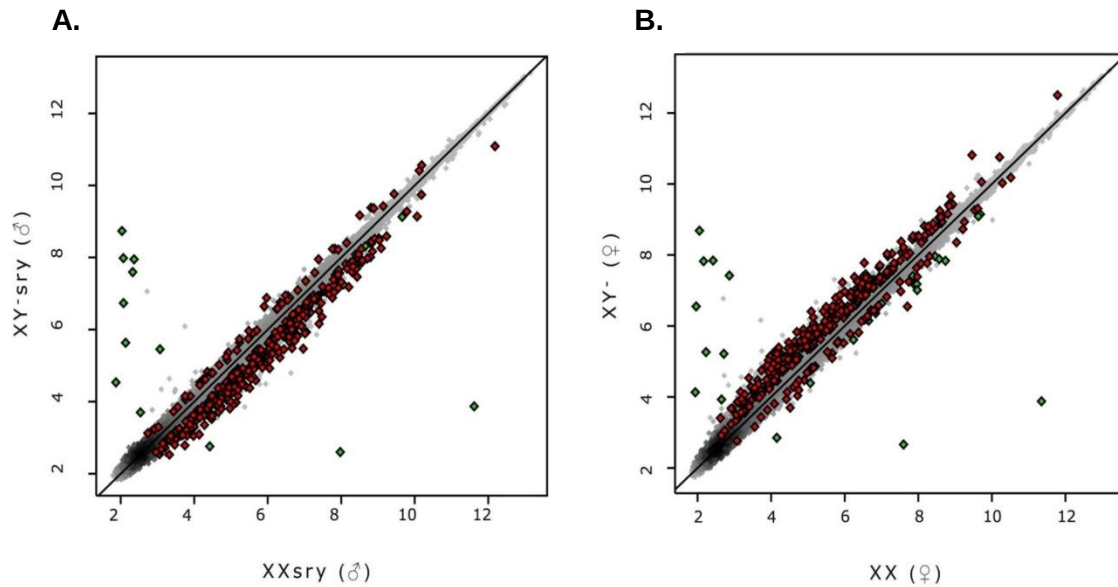


Figure 4.4 – Density plots comparing SCS genes in the presence of different sex chromosome complements. Log2 expression values from processed Affymetrix data were imported for all genes. Each grey point represents a single data point for a specific probe. SCS genes are represented in red and X or Y attached controls in green. Black diagonal line indicates fold change of 1 (no difference). **[A] Density plot for XXsry and XY-sry comparison.** Most autosomal SCS genes are expressed higher in XXsry males than in XY-sry males, concordant with the hCD2 study. **[B] Density plot for XX and XY- comparison.** On the contrary of what was observed with hCD2 gene and male comparison presented in [A], most autosomal SCS genes were found to be expressed higher in XY- females than in XX females.

4.2.5 Sry modulates endogenous gene expression in a sex chromosome-specific manner

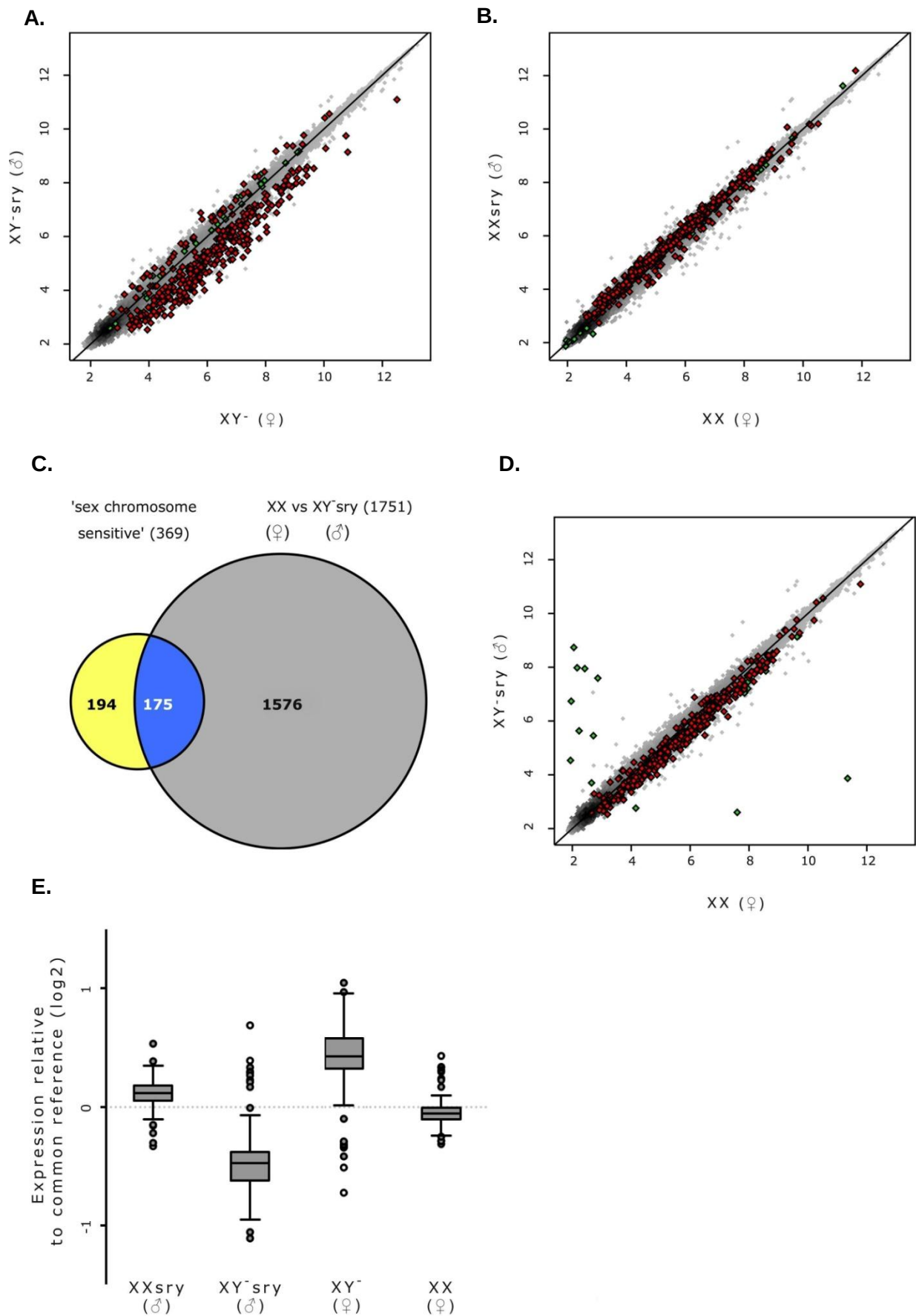
In order to characterise the opposite effect of sex chromosome complement in males (+Sry) and females (-Sry) further, effects of Sry was analysed by comparing males and females with equal sex chromosome complements. Consistent with the idea that Sry might have a repressive effect, the expression levels of 80% of the 369 SCS autosomal genes were found to be significantly higher (Student's t-test, $p < 0.05$; fold change > 1.2) in XY⁻ females than in XY⁻sry males [Figure 4.5A and E]. This suggests that the presence of Sry can indeed have a repressing effect on SCS genes; however, at this stage it is unclear whether this is due to a direct transcription related effect of Sry or an indirect effect mediated by the presence of male sex hormones. Interestingly though, comparison of XX females with XXsry males revealed such repressive effect only happens at a low level in the XX background [Figure 4.5B and E]. This implies that male sex chromosome complement (XY) and Sry are somehow interdependent. Therefore, it was concluded that both Sry and sex chromosome complement play regulatory functions on the SCS genes and therefore may contribute together to sexual dimorphisms. In order to see whether the combined effects of sex chromosome complement differences and the presence of Sry contribute to gene expression differences between genders, SCS genes were directly compared with autosomal genes that are sexually dimorphic in expression levels (Student's t-test $p < 0.05$, fold change > 1.2) between XX females and XY⁻sry males [Figure 4.5C]. Indeed, this analysis revealed that 10% (175 in number) of 1751 sexually dimorphic genes (grey) are also sensitive to sex chromosome complement in males and females (blue). Moreover, an additional group of genes (23%, 401 in number) were found to have gender specific dependence on sex chromosome complement, being expressed differently either in males or females only [not shown]. Finally, the expression of SCS genes were found to be generally higher in XX females than in XY⁻sry males possibly due to combined effects of sex chromosome complement and Sry, which seems to have a repressing action in the presence of Y chromosome only [Figure 4.5D and E].

Overall, it was concluded that sex chromosome interdependent effects of Sry are likely to be responsible for sexual dimorphisms in gene expression, at least on a subset of genes. The effect of Sry was revealed to be highly repressive on this gene set in the XY background, which would otherwise cause an upregulation of SCS genes to an extent beyond the fold change level seen in the normal male (XY-sry) and female (XXsry) comparison. Thus, Sry seems responsible for the SCS genes to be expressed slightly less in normal (XY⁻sry) males compared to normal females (XX).

How does Sry lead to the repression of SCS genes in thymus, which is a somatic tissue? The general idea is that Sry is only expressed during early gonadal development [mouse, 11-11.25 days post coitum; and specifically in the gonadal ridge (Hiramatsu *et al.*, 2009)]. However, Sry mRNA has been detected in foetal and adult mouse tissues including testis, brain, liver, thymus, spleen, heart and lung (Clepet *et al.*, 1993). It was also detected in adult human tissues, primarily in brain (Mayer *et al.*, 1998). Unlike human though, Sry transcripts in mice can form circular conformations [possibly due to (CAG)_n tracts within the exon] and therefore these transcripts could not be translated to protein (Capel *et al.*, 1993). This was thought to restrict the effects of Sry after the important developmental window in the mouse sertoli cells. Interestingly, circular transcripts seem to be specific to the genital ridge as only linear transcripts were detected in the brain of adult mice (Lahr *et al.*, 1995; Mayer *et al.*, 2000). Indeed, Sry is directly regulated in different compartments of adult mouse brain and some brain precursor genes were shown to be direct targets of Sry (Bradford *et al.*, 2009; Dewing *et al.*, 2006). Also, knockdown of Sry in mouse brain via RNAi methods, resulted in motor deficits in male mice (Dewing *et al.*, 2006). Together, these findings reinforce the notion that Sry can indeed affect gene regulation and may interfere with various pathways not only in the genital ridge but also in adult tissues.

Still, in order to figure out whether Sry protein is present in adult mouse thymus, western blots were performed using a commercially available monoclonal Sry antibody (i.e. Abcam, ab22166) as well as a non-commercial polyclonal antibody kindly provided by Peter Koopman's laboratory [The University of Queensland, Australia] (Bradford *et al.*, 2009; Bradford *et al.*, 2007; Wilhelm *et al.*, 2005). As opposed to what was promised by the company guidelines, commercially available antibody gave many multiple bands both in wild type male and female mice thymuses and no specific Sry band was distinguishable [not shown]. In the case of non-commercial αSRY antibody, it seemed that the antibody was cross-reacting with another protein in females and males [see supplementary figure S.13]. Again, no Sry specific band was detected. It was thought that the antibody might cross-react with one of the Sox proteins, which show high homology with Sry [see introduction 4.1.6]. It was also noted that the original study where many Sry antibodies were tested, no female control was included (Bradford *et al.*, 2007). Still, Sry mRNA was detected in male mouse thymus [see supplementary figure S.13] and the presence of Sry protein could not be excluded. Therefore, it is likely that Sry can have a direct effect on SCS genes in adult mouse thymus.

Figure 4.5



See next page for figure legend.

Figure 4.5 - Sry and/or sex modulates gene expression in a sex chromosome-specific manner.

A, B and D are density plots for comparing transcription expression profiles at probe set level. Each grey dot represents a gene, 369 SCS genes are represented with red colour whereas X- and Y- linked control genes are represented with green colour. Gene expression levels were plotted in log₂ and black diagonal line indicates fold change of 1 (no difference). **[A] Density plot comparing XY⁻ females with XY⁻sry males.** Most autosomal SCS genes are expressed higher in XY⁻ females compared to XY⁻sry males. **[B] Density plot comparing XX females with XXsry males.** In contrast to XY- background, the presence of Sry does not have a significant effect on the expression of SCS genes. **[C] Venn diagram showing the overlap between SCS genes and sexually dimorphic autosomal genes.** Gene lists were obtained using data presented in figure 4.2 with p<0.05 (Student's t-test) and fold change > 1.2. The comparison with 1751 autosomal dimorphic genes (grey) with 369 SCS genes (yellow) identified that 175 (10%) of all dimorphic genes are also sex chromosome complement sensitive. **[D] Density plot comparing 369 SCS genes in XX females and XY⁻sry males.** Most autosomal SCS genes are expressed higher in XX females than in XY⁻sry males. **[E] Box-plot comparing expression levels of SCS genes in four core genotype.** Autosomal SCS genes were compared directly using a common reference (average of all four genotypes). Note that the gender/Sry effect is much bigger in mice with XY background rather than XX background. Dotted line indicates reference baseline. [Boxplot was drawn using the R Bioconductor.]

4.2.6 Heterochromatin links of sex-chromosome complement effects and position effect variegation (PEV)

The reporter transgene hCD2 is subject to position effect variegation (PEV) and figure 4.1 presented evidence that its variegation patterns are significantly influenced by sex chromosome complement using four core genotype mice. As explained before, the hCD2 variegation is also highly sensitive to the dosage of heterochromatic factors such as HP1 β (Festenstein *et al.*, 1999; Hiragami-Hamada *et al.*, 2009). Although HP1 has three isoforms and its presence was sometimes linked to gene activation, the β -isoform has been linked mainly to heterochromatin-mediated silencing [see general introduction 1.3.3.4]. Now that a set of sex chromosome sensitive genes had been defined, it was wondered whether such heterochromatic links could provide an explanation for sexual dimorphisms in autosomal gene expression by extrapolation from the initial observation in hCD2 PEV. In order to address this, advantage was taken from a set of microarray data which was produced formerly in Richard Festenstein's laboratory (by Patrick Wijchers, unpublished data generated in MRC CSC-London) using HP1 β over-expressing female XX mice. Here, 450 endogenous genes were shown to be downregulated ($p < 0.05$ Students t-test, fold change > 1.2) in thymus tissue in the case of HP1 β over-expression. Using this data set, it was asked whether this HP1 β -sensitive group of genes were enriched in the sex chromosome complement-sensitive gene set. Indeed, comparison of both groups revealed that 45 (12.2%) of 369 sex chromosome complement-sensitive genes were also sensitive to HP1 β [Figure 4.6A]. An enrichment score of 3.81 ($p < 0.000001$) was calculated against the T cell transcriptome background using Fisher's exact test of over-representation (Dennis *et al.*, 2003; Huang da *et al.*, 2009a, b).

So far, results presented regarding the sex chromosome complement phenomenon on endogenous gene expression were obtained from RNA extracted from a population of cells and could therefore not distinguish between: 1) changes in expression due to all cells behaving similarly or 2) changes in expression being due to a shift in the proportion of cells expressing the affected gene. The latter possibility, at least for a subset of genes, was strengthened by the observations that variegation patterns of the hCD2 transgene are sexually dimorphic and that the SCS gene set is enriched in HP1 β downregulated genes. In order to address this possibility, flow cytometry analyses were performed on the thymocytes of four core genotype mice to determine expression at the level of individual cells. For this purpose, several SCS gene products, which are also sensitive to HP1 β according to figure 4.6A, were tested using specific antibodies. These included the RNA binding protein Msi2, casein kinase Csnk2a1 and the cell surface glycoprotein CD93. Cytoplasmic proteins were

stained with an intracellular FACS staining protocol using 0.05% saponin. No permeabilisation was performed for the staining of cell-surface proteins. At least 20,000 CD4 and CD8 double-positive thymocytes (review-Rothenberg and Taghon, 2005) were selected for testing the subject protein. Four SCS genes examined did not show any variegation patterns [data not shown]. However; the expression of another gene (*Kremen1*), which was not in the original list of SCS genes as the change in expression was below the threshold used in that analysis (see below), exhibited a PEV pattern in a sex chromosome complement-sensitive manner [Figure 4.6B, left and middle panel]. *Kremen1* encodes a transmembrane receptor that functionally co-operates with the ligand DKK1 in the blockage of WNT/ β -catenin signalling, which is crucial for gonad development and speculated to be important for sexual dimorphisms (Liu *et al.*, 2009b; Mao *et al.*, 2002; Nakamura *et al.*, 2001; review-Sekido and Lovell-Badge, 2009; Vainio *et al.*, 1999). Interestingly, *Kremen1* resides on the A1 dark band of mouse chromosome 11 in a place relatively close to pericentromeric repeats [figure 4.6B, right upper panel]; such a pericentromere-proximal location would be expected to render genes more likely to variegate as is the case in hCD2 PEV (Festenstein *et al.*, 1996). Moreover, *Kremen 1* was shown to be HP1 β sensitive, according to the unpublished microarray data. Consistently, FACS analysis of *Kremen1* expression revealed a tri-modal population which is typical for allele specific gene expression patterns. Here, cells that express the *Kremen1* gene on both alleles would exhibit the highest *Kremen1* fluorescence and such a population can be seen as a shoulder on the right-hand side of the histogram. The sharp peak in the middle represents cells likely to have mono-allelic *Kremen1* expression, whereas the shoulder on the left-hand side is likely to represent cells that turned the gene 'off' on both alleles. It is also notable that mean fluorescence levels were similar, suggesting an 'all-or-nothing' effect typical of PEV. Importantly, the percentage of *Kremen1* non-expressing cells correlated well with the sex chromosome complement effects described earlier. Therefore, a *Kremen1* mRNA expression analysis by quantitative real time PCR was also performed on the mouse thymus of four core genotype mice [Figure 4.6B, right lower panel]. Consistent with the flow cytometry analysis, mRNA levels were higher in the absence of *Sry* and highest on the XY background, in a similar fashion with the SCS genes. Fold differences were lower than 1.2 which resulted in its exclusion from the microarray analysis presented in figure 4.2.

Overall, results presented here are in line with the hypothesis that heterochromatin mediated mechanisms can contribute to sexually dimorphic gene expression which is effected by sex chromosome complements. Such heterochromatin mediated effects could cause silencing of endogenous genes in a proportion of cells and can introduce sexual dimorphisms in a PEV-like manner.

Figure 4.6

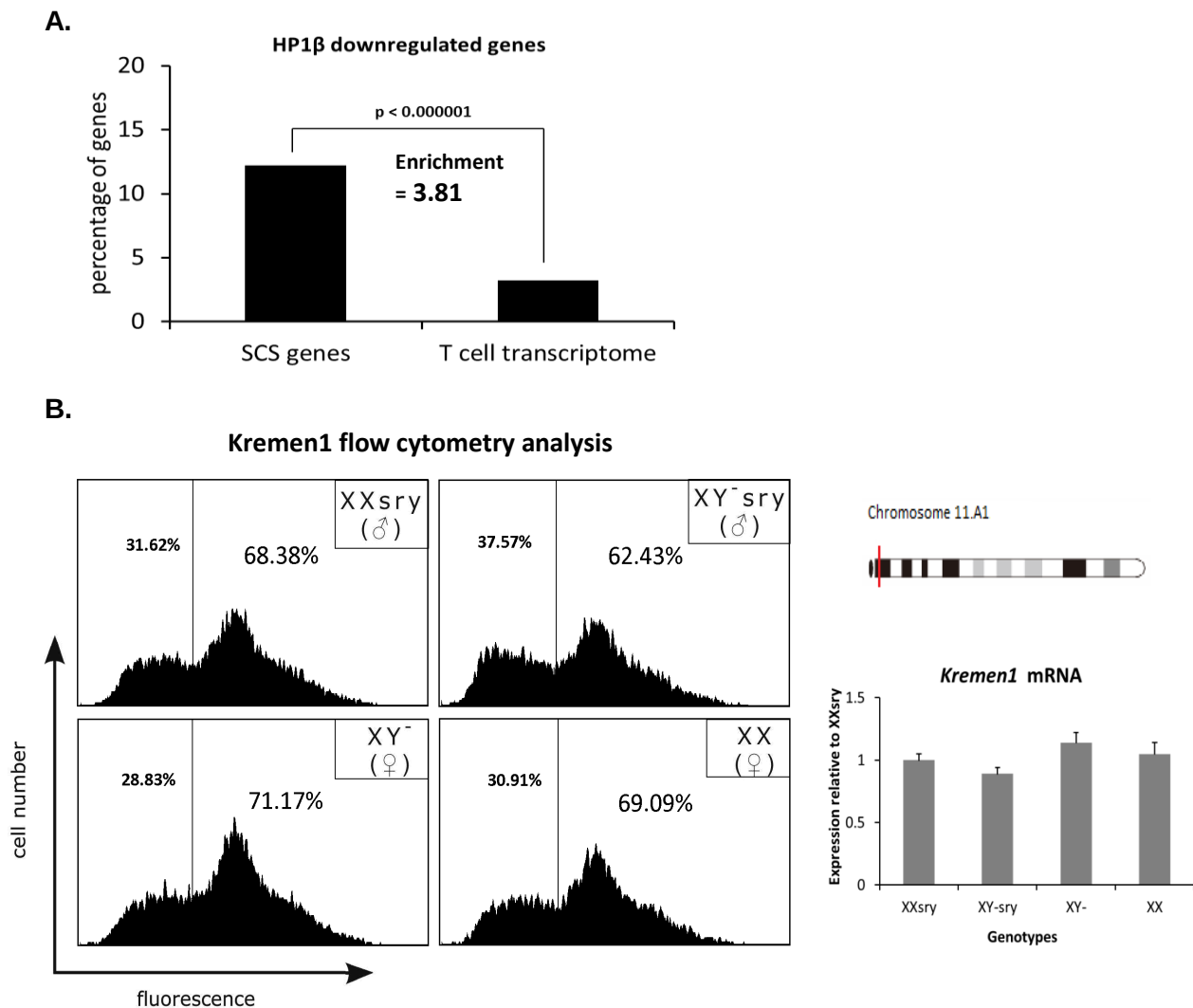


Figure 4.6 – Enrichment of HP1 β sensitive genes among the SCS gene set and PEV-like expression of *Kremen1*. [A] **HP1 β enrichment levels.** Using microarray data obtained from HP1 β over-expressing mice, 45 (12.2%) of the SCS genes were also found to be sensitive to HP1 β . A comparison with the T cell transcriptome revealed an enrichment score of 3.81 with p value < 0.000001 according to Fisher's exact test of over-representation. [B] **FACS and mRNA expression analysis of *Kremen1* on thymocytes.** The expression of *Kremen1* variegates (left and middle panel) and the percentages of non-expressing thymocytes (CD4 and CD8 double positive) correlate well with the sex chromosome complement effect described in figure 4.5. FACS result is consistent with the mRNA levels as detected by Q-RT-PCR (right lower panel). Notably, *Kremen1* resides on a chromosomal location (right upper panel, marked with red) relatively close to pericentromeric repeats. [FACS results are representative for three independent experiments by using three animals per genotype. Expression level of *Kremen1* mRNA was presented relative to XXsry. Normalisation factor for Q-RT-PCR analysis was calculated as the average of the expression of two housekeeping genes, β -actin and *Hmbs*. Error bars represent SEM from three individual animals.]

4.2.7 Response to HP1 β is affected by sex chromosome complement

Figure 4.6 presented a bioinformatics analysis which suggested that heterochromatin protein HP1 β may be involved in the sex chromosome complement effects as the HP1 β sensitive genes were enriched within the SCS gene set. Interestingly, an intriguing HP1 effect was previously reported in *Drosophila*, where conditional depletion of HP1 α influenced a significantly higher number of genes in male flies than in females and caused male-sex biased chromosomal defects as well as preferential lethality (Liu *et al.*, 2005). In order to characterise the sexually dimorphic effects of HP1 in mouse and to see whether these were dependent on sex chromosome complement, XY^{sry} males (C57BL/6 background) were crossed with *HP1 β* over-expressing XX females (CBA.ca) (Festenstein *et al.*, 1999). Thymuses dissected from the resultant progeny (6-8 weeks old) with different sex chromosome complements underwent RNA extraction followed by cDNA synthesis and were analysed with Q-RT-PCR using primers specific to 23 SCS genes which are also HP1 β sensitive according to the data presented in section 4.2.6 [Figure 4.7A]. The expression levels of these 23 SCS genes in *HP1 β* wild type mice (black columns) corresponded well with what was presented in figure 4.6; thereby independently confirmed the sex chromosome complement effects presented in figure 4.5. Over-expression levels of HP1 β (3-4 fold) were found consistent with what was described before [Figure 4.7B] (Festenstein *et al.*, 1999). Interestingly, the response of SCS genes to HP1 β seems to change direction depending on sex chromosome complement [Figure 4.7A]. *HP1 β* over-expression slightly downregulated SCS genes on both XX and XY backgrounds in the presence of *Sry*. The same effect was also observed for XX female mice; this is consistent with the *HP1 β* microarray data mentioned in figure 4.6A. Surprisingly though, SCS genes were upregulated in the case of HP1 β over-expression in the XY females, where they are already expressed at a higher level in comparison with HP1 β wild type mice (black columns). Notably, the expression status of the housekeeping genes *Hprt* and *Hmbs* was found to be unaffected by both sex chromosome complement or the dosage of HP1 β [Figure 4.7C]. This intriguing result further strengthened the hypothesis that heterochromatin mechanisms may underlie sexual dimorphisms as the direction of the response to HP1 β was dependent on sex chromosome complement. Importantly, *HP1 β* over-expression seems to accentuate the normal expression levels (*HP1 β* wild type) of SCS genes. This is particularly obvious in the XY⁻ background where the response of SCS genes, which are already higher in HP1 β wild type mice, was upregulation in contrast to other backgrounds. This observation implicates HP1 β in the establishment of sexual dimorphism in the expression of a sub-set of autosomal genes. In other words, the repressing effect of *Sry* on SCS genes [presented in figure 4.5] could be mediated by HP1 β and regulated by sex chromosome complement.

Figure 4.7

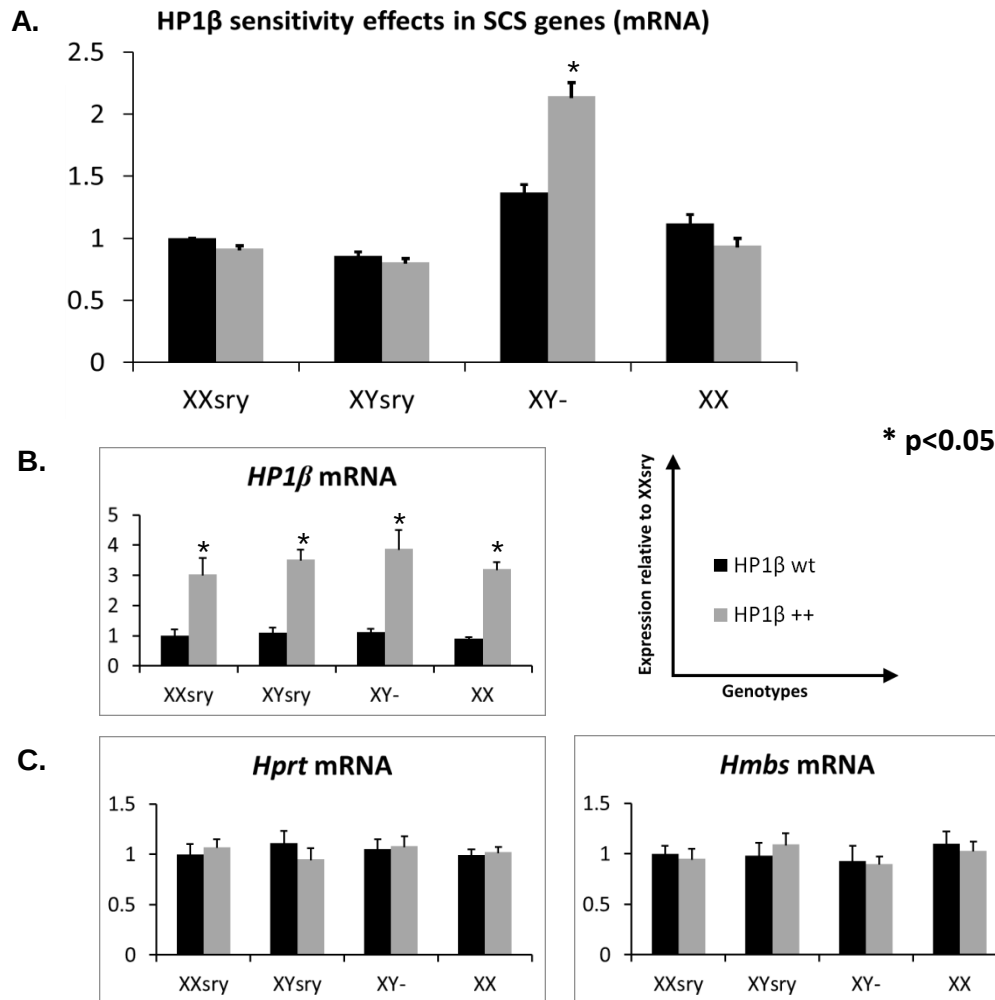


Figure 4.7 – HP1 β sensitivity effects on 23 SCS genes in four core genotype mice. Levels of mRNA were analysed level using specific primers against 23 SCS genes that are also HP1 β sensitive according to microarray data mentioned in figure 4.6. Expression values are relative to XXsry. The normalisation factor for the Q-RT-PCR analysis was calculated using the average of expression levels of two housekeeping genes *Hmbs* and β -actin. Error bars represent the standard error of the mean from three different animals. **[A] Expression levels (mRNA) of 23 SCS genes under HP1 β over-expression.** mRNA levels of SCS genes in HP1 β wild type mice correlated well with what was presented in figure 4.5. Over-expression of HP1 β caused a slight downregulation of SCS genes in all backgrounds but XY $\bar{c}$ where the effect seemed to be to accentuate the expression difference. **[B] HP1 β expression levels (mRNA).** 3-4 fold upregulation was seen in HP1 β over-expressing mice. **[C] Expression levels of housekeeping genes.** Both *Hprt* and *Hmbs* expression seems to be unaffected, neither by sex chromosome complement nor by the HP1 β over-expression. Normalisation for C was done using β -actin expression levels only. [* indicates student's t-test in comparison to the control group. The 23 SCS and HP1 β sensitive genes were: 4930402E16Rik, 6820431F20Rik, Ankhd2, Ash1, Atxn2, Csnk2a1, Ddi2, Ddx6, Dyspl3, Eif4ebp2, Elf2, Fryl, Itch, Me2, Myh9, Mll3, Nisch, Otub1, Ptpre, Sf3b1, Sfi1, Sp1, Spnb2.]

4.2.8 Repetitive DNA as a potential regulator of sex chromosome complement effect

Figure 4.6 and 4.7 suggested a link between heterochromatin regulation and sex chromosome complement effect. Heterochromatin is commonly thought to have evolved as a protective response against the possible intruding effects of repetitive DNA sequences and their contribution to transcriptional noise (review-Bashkirov, 2002; Yoder *et al.*, 1997). Many studies in the literature reported that both transposable elements and satellite repeats play vital roles in the regulation of heterochromatin formation [see general introduction 1.8]. Whereas classical repeats such as minor and major satellites are almost solely associated with heterochromatin (Festenstein *et al.*, 2003; Festenstein *et al.*, 1996; Guenatri *et al.*, 2004; Martens *et al.*, 2005; Peters *et al.*, 2001), the role of transposable elements seem to differ based on the type of the transposon or the host cell type (Ganapathi *et al.*, 2005; Martens *et al.*, 2005; review-Zamudio and Bourc'h, 2010). Interestingly, about a quarter of human promoters, and a significant proportion of matrix attachment regions (MARs) and locus control regions (LCRs) were found to be derived from transposon-derived sequences (Jordan *et al.*, 2003). Moreover, 4% of active genes also have transposable elements within their coding regions (Nekrutenko and Li, 2001).

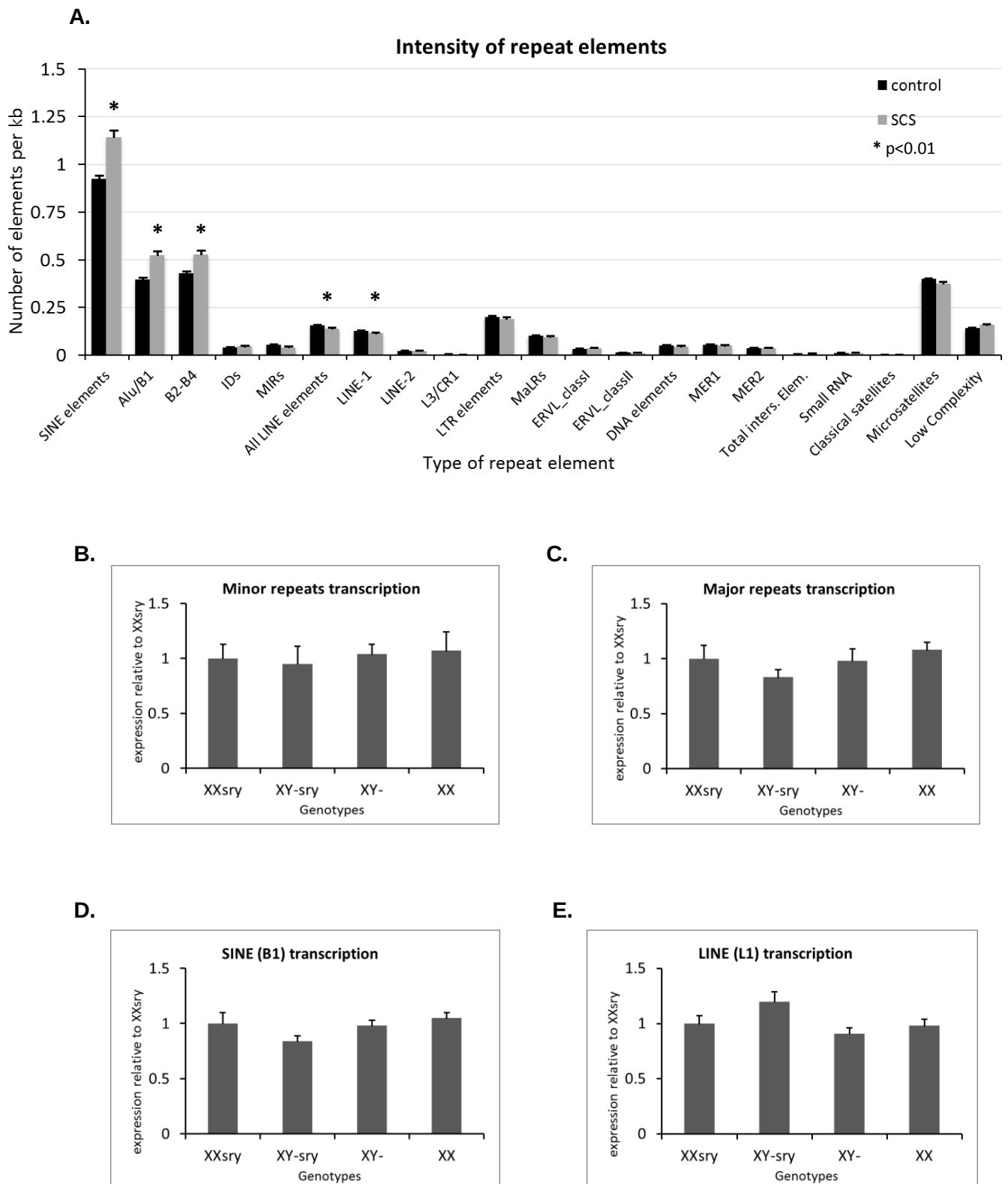
In order to determine whether there were any prominent repetitive DNA sequences around the SCS genes, the sequences of all 369 SCS genes along with 10 kb flanking regions on both sides were analysed using the RepeatMasker bioinformatics software (version 3.2.9) [<http://www.repeatmasker.org/>; (Tarailo-Graovac and Chen, 2009)]. A comparison of repeat element intensity (as elements per kb) between the 369 SCS genes and randomly selected 1576 genes (using MS Excel random function) is presented in figure 4.8A. Accordingly, types of SINE elements (B1-4) were found to be enriched in SCS gene loci compared to the control group ($p < 0.01$, Kolmogorov-Smirnov test). On the other hand, intensity of LINE elements were found to be slightly lower in the SCS group ($p < 0.01$, Kolmogorov-Smirnov test). Interestingly, the extent of difference in the LINE and SINE intensity levels are around the 1.2 fold threshold, corresponding well with the transcription levels of SCS genes as presented in figures 4.5 and 4.7. RepeatMasker analysis also checked occurrence levels of LTR retrotransposons, DNA transposons as well as small RNA encoding regions, and none of them were shown to be enriched within the SCS gene loci. Very few classical satellite repeats were detected both in control and SCS gene loci; hence no proper comparison could be made. This is consistent with the fact that classical satellites typically do not occur in gene rich regions [see general introduction 1.8.3.2]. However, a high number of microsatellites were detected in both groups. A slight difference, which is not statistically

significant, was shown in the intensity of microsatellites within the SCS group. Finally, low complexity repeats such as poly-purine/pyrimidine stretches, or regions of high AT or GC content were shown to exist at a relatively higher level in SCS genes without statistical significance.

In order to see whether there is a direct effect of sex chromosome complement on the expression levels of repeat elements, RNA was extracted from the thymuses of 6-8 week old four core genotype littermates followed by cDNA synthesis and Q-RT-PCR analysis using specific primers for several repeat elements [same primers with (Martens *et al.*, 2005)]. Centromerically located minor satellites were found to be expressed at the same level in all genotypes [Figure 4.8B]. On the other hand, pericentromeric major repeats seem to be affected by sex chromosome complement [Figure 4.8C]. Major repeats are known to be universally suppressed by heterochromatin and this is thought to be essential for the maintenance of chromatin architecture [see general introduction 1.8.3.2]. Therefore, any difference in the expression levels of these repeats may be a result of influence of sex chromosome complement on heterochromatic silencing mechanisms. Pericentromeric major repeats were found to be expressed at a lower level in the presence of the Y chromosome but only in the presence of Sry suggesting a repressive role of Sry specifically in the presence of the Y chromosome. In addition, expression levels of SINE elements were also checked and the pattern resembles that of the major repeats but with a less pronounced effect [Figure 4.8D]. Still, SINE expression was lowest in the XY^{sry} background. On the contrary though, expression of LINE elements was higher in the presence of Sry, particularly in the XY-sry background [Figure 4.8E]. Again, differences in the expression levels for all tested repeat elements are around the 1.2-fold level. Although this is a small difference, it fits well with the fact that such small differences of gene expressions seem to regulate sexual dimorphisms (Yang *et al.*, 2006).

Overall, both RepeatMasker analysis of SCS genes and expression analysis of repeats suggested that repetitive DNA, particularly SINE and LINE elements could potentially contribute to the sex chromosome complement effect described so far. Q-RT-PCR results of minor repeats have implications for possible sex chromosome complement related differences between the heterochromatin mediated silencing mechanisms [see discussion 4.3.4].

Figure 4.8



See next page for figure legend.

Figure 4.8 – Analysis of repetitive DNA within the context of sexual dimorphism.

[A] RepeatMasker analysis. Here, the intensity of repeat elements (as elements per kb) was analysed both on 369 SCS genes and also on a control group comprising randomly selected 1576 genes. The analysed gene loci included gene bodies as well as 10kb flanking regions both upstream and downstream. Intensity of SINE elements are enriched in the SCS group whereas an opposite result was obtained for LINE elements. Columns marked with asterisk (*) are statistically different from the control group based on parameter and distribution free Kolmogorov-Smirnov test.

[B] Transcription levels of minor repeats. No difference was detected among the four core genotypes.

[C] Transcription levels of major repeats. Pericentromeric major repeats are expressed at a lower rate in the presence of Sry, particularly in XY^{sry} background.

[D] Transcription levels of SINE (B1) elements. In a similar fashion with major repeats, B1 transcription is lowest in the XY^{sry} background.

[E] Transcription levels of LINE (L1) elements. In contrast to [C] and [D], expression of L1 is highest in the XY^{sry} background. [Expression values are relative to XX^{sry}. The normalisation factor for the Q-RT-PCR analysis was calculated using the average of expression levels of two housekeeping genes *Hmbs* and *β-actin*. Error bars represent the standard error of the mean from three different animals.]

4.3 Discussion

4.3.1 'Sink' effect hypothesis for sexual dimorphisms in heterochromatin

The direct contribution of sex chromosomes to epigenetic sex differences is largely unknown. However, several potential mechanisms could be hypothesised based on the findings presented in this thesis and some previous studies. Since the expression of the hCD2 transgene is particularly sensitive to heterochromatin related histone marks such as increased H3K9me3 and reduced acetylation levels as well as DNA methylation and HP1 (Festenstein, 1996; Festenstein *et al.*, 2003; Festenstein *et al.*, 1999; Hiragami-Hamada *et al.*, 2009), such factors could underlie the finding that heterochromatic silencing of the hCD2 gene is decreased in the presence of an extra X chromosome. Importantly, it is likely that the inactivated X chromosome may act like a 'sink' that captures a high concentration of heterochromatin related factors and thereby reduces their chances of recognising autosomal genes (Chadwick and Willard, 2003c). Such 'sink' effects were observed before in *Drosophila*, where the presence of an extra Y chromosome, which is mainly heterochromatic, reduced PEV of autosomal genes [see general introduction 1.5.2] (Dimitri and Pisano, 1989; Gowen and Gay, 1934; Lemos *et al.*, 2010).

The inactive X chromosome is particularly enriched in repressive histone marks such as H3K9 and H3K27 methylation as well as DNA methylation, and exhibits considerably less H3/H4 acetylation [see introduction 4.1.5]. Although further scientific evidence is needed in characterising the 'sink' effect in mammals, one can therefore expect that the presence of an inactivated X chromosome in females might titrate away heterochromatin related factors such as HKMTs, HDACs, PRCs or HP1s. In line with this, former studies reported reduced DNA methylation levels associated with the XX background. Mouse embryonic stem cells on a XX background display global DNA hypomethylation compared with XY and XO ES cells (Zvetkova *et al.*, 2005). Similarly, mitotically inherited DNA methylation patterns in embryonic stem cells lacking DNMT3 are lost at a faster rate in the XX background compared to the XY background (Ooi *et al.*, 2010). Moreover, primordial *ex vivo* germ cells exhibit DNA hypomethylation associated with XX background (Durcova-Hills *et al.*, 2004; Durcova-Hills *et al.*, 2006). Finally, induced pluripotent stem cells derived from mouse embryonic fibroblasts also revealed reduced DNA methylation levels at female centromeres (Maherali *et al.*, 2007). Although DNA hypomethylation on the XX background was linked to reduced levels of autosomally expressed Dnmt3a and Dnmt3b proteins in the study of Zvetkova *et al.*, Ooi *et al.* reported no change in the expression levels of these proteins and thereby these results could be a reflection of a 'sink' effect for Dnmts in the XX background.

4.3.2 SRY and HP1 as primary drivers of sexual dimorphism on gene expression

Results in this study concluded that the presence of *Sry* has a repressing effect on hundreds of sex chromosome complement-sensitive genes as they are expressed higher in the absence of *Sry* on both XX and XY⁻ backgrounds compared to XX^{sry} and XY^{sry} [Figure 4.5E]. However this effect seems to be largely dependent on sex chromosome complement as the repressing of SCS genes in the presence of *Sry* was particularly boosted in the XY background, whereas a lower repressing effect could be seen on the XX background. This suggests that *Sry* possibly co-operates with factors specific to the Y chromosome, as recently suggested (review-Arnold, 2011). Interestingly, upregulation of genes in the absence of *Sry* was shown to occur to a greater extent in XY⁻ females compared to XX females. This suggests that *Sry* protein might have acquired an evolutionary role where it compensates the expression levels of SCS genes, bringing them to almost complete balance between the sexes [Figure 4.4D]. Such a 'compensatory' role for *Sry* is consistent with an evolutionary hypothesis that sexual dimorphisms dependent on sex chromosome complements may introduce compensatory mechanisms (review-De Vries, 2004).

How can *Sry* mediate a repressing activity, although it is better known as a transcriptional activator [see introduction 4.1.6] ? The answer might be provided by its functional interaction with Kruppel-associated box (KRAB) containing transcription factors [Figure 4.9] which can simultaneously interact with TIF1 β (also known as KAP1 or Trim28) (Oh *et al.*, 2005; Peng *et al.*, 2009; Polanco *et al.*, 2009). Importantly, TIF1 β recruits HP1 isoforms (Cammass *et al.*, 2000; Nielsen *et al.*, 1999; Ryan *et al.*, 1999) as well as the H3K9 specific methyltransferase SETDB1 (Schultz *et al.*, 2002) and HDAC transcriptional co-repressor complexes N-CoR (Underhill *et al.*, 2000) and NuRD (Schultz *et al.*, 2001). There are hundreds of KRAB-domain-containing proteins identified in mammals (Huntley *et al.*, 2006; Krebs *et al.*, 2005). Whereas some of these only contain the KRAB domain (KRAB-O), many others have a DNA recognising zinc finger motif (KRAB-ZF). According to Oh *et al.* and Peng *et al.*, the SRY bridge domain (BRG) can interact both with KRAB-O and KRAB-ZF but to a greater extent with KRAB-O. It is thought that SRY could ensure the stability of the KRAB-TIF1 β -HP1 silencing complex in the case of KRAB-zinc fingers, which recognise their own targets [Figure 4.9A]. On the other hand, DNA sequence binding can be maintained by SRY in the case of KRAB-O [Figure 4.9B]. Interestingly, two KRAB-ZF proteins (i.e. regulator of sex limitation; RSL factors) were shown to control sexually dimorphic gene expression in mouse liver (Krebs *et al.*, 2003). Together with the data presented in figure 4.5, it is tempting to speculate that SRY might directly repress the large group of genes that are affected by sex chromosome complement.

Another intriguing finding described in this thesis is the enrichment of HP1 β sensitive genes among the SCS group and the accentuated effect on SCS gene expression as a response to *HP1 β* over-expression [Figures 4.6 and 4.7]. Interestingly, *HP1 β* over-expression upregulated SCS genes only in the XY⁻ females as opposed to other three backgrounds, implying that the presence of Sry is needed in order to downregulate SCS genes in the presence of the Y sex chromosome complement. Although HP1 is a dominant gene silencer, some transcription activating roles were also reported especially for HP1 γ and in rare cases for HP1 β [see general introduction 1.3.3.4]. Although the context and mechanisms in relation to this phenomenon remain to be elucidated, post-translational modifications of HP1 were proposed to modulate HP1 function [see general introduction 1.3.3.4]. In this respect, enzymes responsible for post-translational modification, such as kinases and phosphatases, which are encoded by sex chromosomes, should be evaluated. Interestingly, cyclin dependent kinase Pctk1 is a human XCI escapee that does not have an apparent Y homologue (Carrel *et al.*, 1996). Moreover, a serine threonine kinase PrkX and its homologue PrkY could have different expression levels or different targets between males and females, as exemplified by *Utx/Uty* [see section 4.3.3].

The gene ontology analysis of SCS genes [Figure 4.3] revealed many transcription factors as well as some chromatin related factors. It could be speculated that downstream players of the sex chromosome complement and Sry effect described here could be within this group. These include genes that encode H3K4 specific methyltransferases Mll -3 and -5 , Ash1l (Kim *et al.*, 2006) as well as Whsc1l1 which also has a methyltransferase activity on H3K4 and H3K27 residues (Tsukada *et al.*, 2006); and a H3K36 demethylase Jhdm1 (Kdm2a, Fbxl11) (Wysocka *et al.*, 2006). Moreover other proteins which associate with important chromatin factors are encoded by genes within the SCS set. These are Bptf, which is a PHD finger transcription factor that recognises methylated H3K4 residues and recruits the nucleosome remodelling complex, NURF (Chiba *et al.*, 1994); Smarca4, a component of the SWI/SNF remodelling complex and associates with hormone receptors such as estrogen and retinoid acid receptor (review-Ooi and Wood, 2007); COREST, which is a co-repressor that interacts with many chromatin factors including HDAC1 and HDAC2 as well as the histone demethylase LSD1 (Barak *et al.*, 2003; Zhang and Dufau, 2002; review-Zhang and Dufau, 2003); and retinoblastoma binding protein Rbbp4, a WD-repeat protein often found in co-repressor complexes such as Sin3 and NuRD (Oh *et al.*, 2005).

Figure 4.9

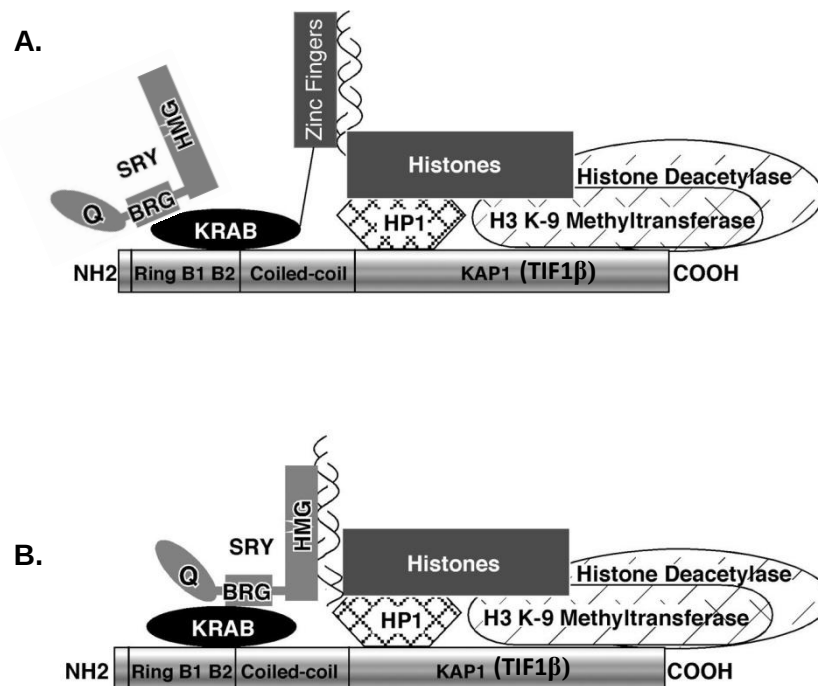


Figure 4.9 – Proposed models for SRY mediated repression. SRY interacts with KRAB domain containing proteins, a common motif to many transcription factors. KRAB domain proteins can simultaneously interact with the transcriptional co-repressor TIF1β, which in turn interacts with HP1 isoforms, H3K9 methyltransferases as well as histone deacetylase silencing complexes. **[A] SRY interaction with KRAB-zinc finger proteins.** Here, KRAB-ZF factor recognises target genes and SRY may have a structure stabilising role on the overall silencing complex. **[B] SRY interaction with KRAB-O proteins.** In this case, SRY recognises target DNA sequences by its conserved HMG box. As a result of the interaction with KRAB-O, TIF1β can be recruited which attracts HP1, H3K9 methyltransferases and HDAC complexes to the gene locus in order to repress transcription. [Figure adapted from (review-Helena Mangs and Morris, 2007).]

4.3.3 Sex chromosome linked genes as possible regulators of sexual dimorphisms

Although the ‘sink’ effect hypothesis fits well with the de-repressing effect of the second X chromosome on the silencing of the hCD2 transgene [Figure 4.1], the situation seems to be more complex for most of the autosomal genes [Figures 4.4 and 4.5]. Accordingly, both the sex chromosome complements and the presence of Sry have combined regulatory effects on SCS genes. In order to understand the mechanisms behind direct sex chromosome complement effects and their interaction with Sry, possible factors encoded by genes residing on sex chromosomes should be evaluated.

The X chromosome contains more than 800 protein-coding genes in mouse and human, whereas the smaller Y chromosome only harbours 48 protein-coding genes in human and 12 in mouse [based on Ensembl release 59, see figure 4.10]. A high number of Y-chromosome genes reside in the pseudoautosomal regions (PAR) which display sequence identity on both sex chromosomes. Genes within the PAR have the ability to recombine during meiosis and therefore are typically the same on the X and Y chromosomes (Skaletsky *et al.*, 2003; Toure *et al.*, 2005). However, PARs only represent 5% of the Y chromosome and the remaining region is known as the “male specific region of the Y (MSY)” , which cannot recombine with the X chromosome and contains highly repetitive sequences with only few protein coding genes and many pseudogenes (review-Graves, 2006). Although genes in the MSY are specific to males, many human MSY genes are known to have homologues on the X chromosome and are ubiquitously expressed. The remaining non-homologous genes are mainly involved in sex determination and spermatogenesis (Ellis *et al.*, 2005; Mueller *et al.*, 2008; Reynard *et al.*, 2007; Toure *et al.*, 2005). Interestingly, *Sycp3*-like Y-linked gene (*Sly*) is present in many more copies in the MSY compared to its X homologue *Slx*, which is thought have both cytoplasmic and chromatin related functions during spermatogenesis (Cocquet *et al.*, 2009). shRNA mediated knockdown of *Sly* caused global de-repression of dozens of male germ line specific genes both on sex chromosomes and autosomes (Reynard *et al.*, 2009). This is thought to be because of chromatin related functions of *Sly*, which interacts with histone acetyltransferase Tip60 probably having an inhibitory role on the action of the complex (Xu *et al.*, 2008a; Xu *et al.*, 2008b).

As explained in 4.1.5, some genes on the X chromosome are known to be inactivation escapees, some of which have homologues on the Y chromosome ensuring a balance in dosage between males and females [Figure 4.10, blue]. However, even in the case of a Y homologue, the expression levels of such escapees are not always sufficiently balanced. For example, two known XCI escapees, *Utx* and *Jarid1c* that encode H3K27 and H3K4 specific

histone demethylases respectively, were reported to be expressed more in the female brain compared to male brain, where their Y counterparts *Uty* and *Jarid1d* could not compensate this difference (Hong *et al.*, 2007). Importantly, although *Utx* has an H3K27 specific action (Lan *et al.*, 2007) *Uty* does not seem to perform any H3K27 specific demethylase activity (Ditton *et al.*, 2004). This suggests that homologous genes on sex chromosomes might have diverged during evolution and acquired specialised functions. In the case of *Utx/Uty*, one may expect that expression and function related differences may contribute to sexual dimorphisms on chromatin and thereby affect autosomal gene expression.

In addition to these, the Y chromosome homologues may also exhibit differences in the translation of mRNA to protein compared to their X chromosome versions. For instance, the translation of *Ddx3y*, which encodes an RNA helicase, seems to occur only in the male germ line; although the mRNA expression was measured at the same level with its X counterpart *Ddx3x* in many tissues (review-Arnold, 2011; Carrel and Willard, 2005; Johnston *et al.*, 2008; Nguyen and Disteché, 2006; review-Wijchers and Festenstein, 2011). In mouse, there are also several XCI escapees that do not have homologues on the Y chromosome [Figure 4.10, black]. These include cytoskeleton and microtubule related genes *Shroom4* and *Mid1*, carbonic anhydrase encoding *Car5b* and translation initiation factor gene *Eif2s3x* (Cai *et al.*, 2003; Smith *et al.*, 2005). In humans, several chromatin-related factors were also shown to escape XCI without having a homologue on the Y and thereby could drive sexual dimorphisms in gene expression. These include genes that encode HDAC8 and the histone acetyltransferase complex subunits MSL3 and MORF4L2 (Eggan *et al.*, 2000; Mak *et al.*, 2004; Okamoto *et al.*, 2004).

Although combinatory effects of X-inactivation escapees or Y-specific genes are potential targets behind the sex chromosome complement effects in adult tissues, there is a developmental period prior to XCI when both X chromosomes are active at early blastocyst stage and therefore chromatin related factors encoded on the X chromosomes could be expressed at a higher level on the XX background in this short but highly important period [e.g. Histone deacetylases Hdac6, Hdac8, Sin3b; DNA-methyl binding Mecp2; in addition to histone methyltransferases *Utx* and *Jarid1c*] (Carone *et al.*, 2010). Therefore, one may expect that these enzymes might epigenetically mark the genes for further regulatory events to take place at later stages during development.

Finally, imprinting effects could be another dimension that may contribute to sexual dimorphisms in gene expression. In line with this, paternal diet seems to affect the expression of hundreds of genes (Davies *et al.*, 2005; Raefski and O'Neill, 2005). Imprinting can take place both on autosomes and X chromosomes. As a result, the paternally imprinted

allele on the X chromosome could have different expression levels compared to the maternal allele. Such differences were reported for the X-linked lymphocyte regulated (i.e. *Xlr3b*, *Xlr4b*, *Xlr4c*) gene family in mouse (Bergsagel *et al.*, 1994). Xlr proteins are known to be expressed in lymphocytes and their exact function still remains unknown (Gregory *et al.*, 2002). Davies *et al.* and Raefski *et al.* suggested that these are chromatin related factors and are also involved in neurodevelopment with implications for cognitive function. Such imprinting effects could also be possible on other X chromosome genes and remain to be investigated.

Figure 4.10

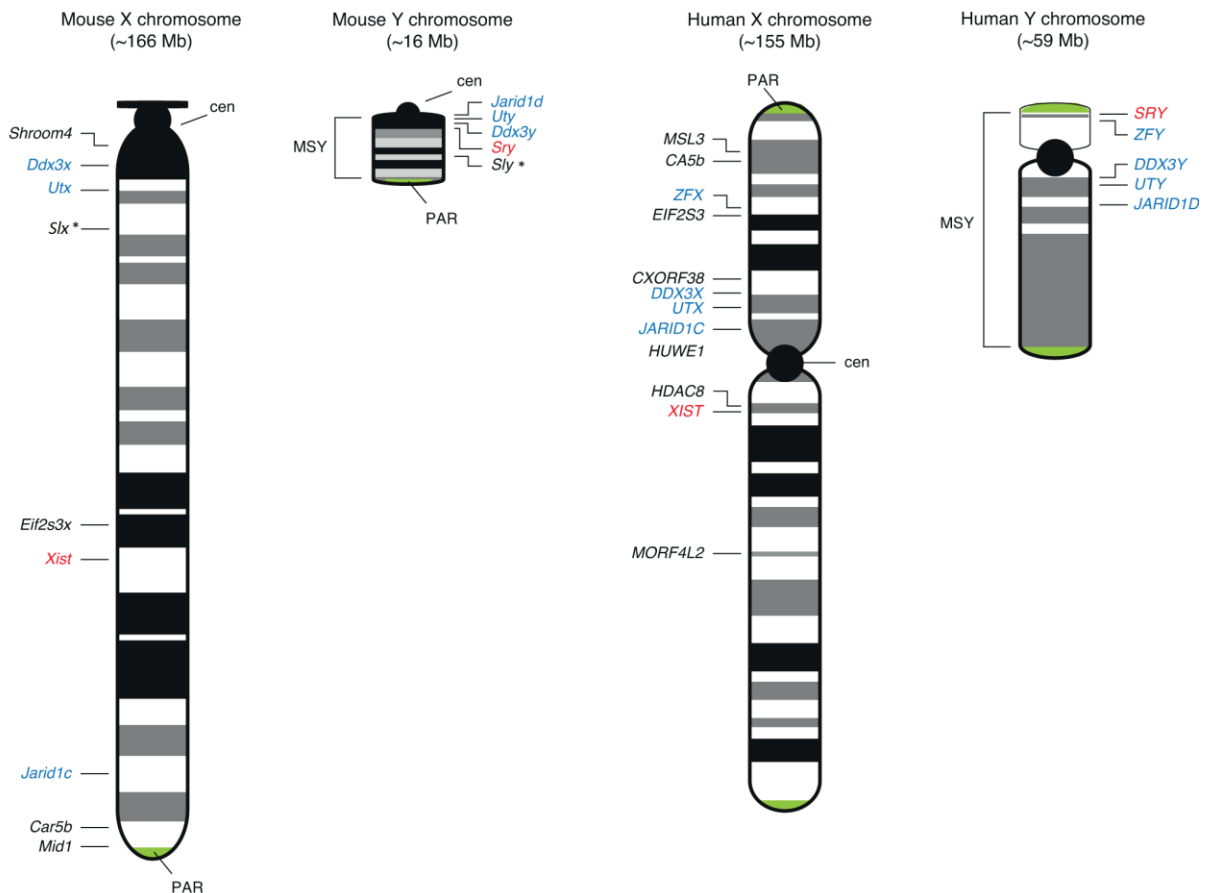


Figure 4.10 – Comparison of X and Y chromosomes. Both mouse and human X chromosomes are larger than Y chromosome. In order to deal with potential imbalances caused by the two copies of X chromosomes in females, one X chromosome is inactivated (Xi) in mammals. Xi still carries some genes that escape the inactivation process and therefore have higher expression levels in females. On the other hand, the Y chromosome contains some Y specific genes, which are potential drivers of male sex development and related features. The most important such gene is SRY which is essential for male gonadal development. Both sex chromosomes have pseudoautosomal regions (PAR) that are targets for recombination in meiosis. These regions comprise many genes which are homologous between males and females. Still, some other genes are not subject to recombination. In males, several such genes are identified in the male specific region of Y (MSY). Male or female specifically expressed genes are in red, genes escaping XCI that have a homologue on the Y chromosome in blue and escapees without an apparent homologue in black. Figures were drawn according to NCBI and ENSEMBL records. It is noteworthy that mouse Y chromosome were reported to be larger (78Mb) elsewhere (review-Wijchers and Festenstein, 2011). *Slx and Sly are homologues, however many other copies of Sly were detected on the Y chromosome [Adapted from (Matsuda *et al.*, 2001; Ryan *et al.*, 1999).]

4.3.4 Repetitive DNA and sexual dimorphism

Figure 4.8 presented both evidence regarding a possible contribution of repetitive DNA sequences to the sexual dimorphisms in autosomal gene expression. Although no difference was detected in the expression levels of centromeric minor transcripts, it was found that pericentromeric major repeats were expressed less on the XY background in the presence of Sry. Although there was more silencing of the hCD2 gene on the XY background, no effect of Sry was seen [Figure 4.1C]. The ‘sink’ hypothesis provides an explanation for the effects observed on the hCD2 PEV. Likewise, the ‘sink’ hypothesis mentioned above could also explain the upregulation of major repeats on the XX background. On the other hand though, the repressing effect of Sry is rather intriguing and elusive. Notably, TIF1 β is localised in pericentromeric heterochromatin in mouse cells (Eller *et al.*, 2007; Ganapathi *et al.*, 2005; Han *et al.*, 2004). Considering the model presented in figure 4.9, it is tempting to speculate that the stability of HP1-TIF1 β silencing complex could be modulated by the presence of Sry on pericentromeric repeats.

RepeatMasker analysis revealed that SINE elements are enriched within the SCS gene loci whereas LINE elements are impoverished [Figure 4.8A]. Interestingly, the expression levels of SINE elements resemble that of major repeats [Figure 4.8C]. On the contrary though, LINE elements are expressed particularly higher on the XY^{sry} background [Figure 4.8D]. Corresponding well with the original hypothesis that gender differences could be determined by differences in heterochromatin mediated silencing secondary to sex chromosome complement [see introduction 4.1.8], it was shown that sex chromosome complement-sensitive genes are indeed affected by heterochromatin as presented in figures 4.6 and 4.7. The enrichment of SINE elements within the SCS gene set was perhaps surprising at first sight as previous studies reported a positive correlation with the intensity of SINE elements and active gene expression (Eller *et al.*, 2007; Ganapathi *et al.*, 2005; Han *et al.*, 2004). At this point, it should be noted that HP1 β downregulated genes determined by previous microarray experiments using *HP1 β* over-expressing mice (by Patrick Wijchers, unpublished data generated in MRC CSC-London) also showed a marked SINE dominance and slight LINE paucity following the same type of analysis. The differences between previous reports and this study could be explained by the fact that the chromatin-mediated regulation of transposable elements might differ according to the cell type and proliferation status (Martens *et al.*, 2005; Thorey *et al.*, 1993; Willoughby *et al.*, 2000). In mouse thymus, data in this thesis already showed that SINE (B1) elements are indeed enriched for the H3K9me3 heterochromatin mark as well as proteasome binding at a level comparable to major satellites [Figure 3.2]. On the other hand, LINE (L1) elements had a relatively low H3K9me3

and proteasome signal, in a manner reminiscent of the active genes. Moreover, although B1 elements are known to be the mouse counterparts of Alu, the average length, repetition and orientation between the mouse and human genomes are different (Lander *et al.*, 2001; review-Richard *et al.*, 2008; Waterston *et al.*, 2002). Therefore, it is possible to conclude that SINE elements are targeted by heterochromatin in adult mouse thymus and contribute to the regulation of SCS genes by increasing the probability of recruiting heterochromatic factors.

How can transposable elements affect the regulation of SCS genes? This could be achieved either by direct chromatin regulatory effects attracted by them or due to their ability to serve as gene promoters. For example, SINE elements were shown to act as heterochromatin barriers in mice albeit in the so far limited context of the *Keratin 18* gene (Graff *et al.*, 1997). On the contrary though, SINE elements were also shown to promote DNA methylation in the genes that encode E-cadherin (E-cad) and the tumour suppressor von Hippel-Lindau (VHL) in mice (Chen *et al.*, 2008). Moreover, inverted Alu repeats seem to introduce silencing effects on a reporter gene in human cells; possibly by causing bi-directional transcription and recruiting RNAi mediated silencing mechanisms (Babich *et al.*, 1999; Norris *et al.*, 1995; Zhou *et al.*, 2002). In addition to these, binding of several transcription factors to sequences within various SINE subfamilies have been reported. These include developmental transcription regulator PAX6, as well as hormone response factors such as estrogen and thyroid receptors for Alu (Allen *et al.*, 2003; Bailey *et al.*, 2000; Lippman *et al.*, 2004). Overall, the enrichment of SINE elements within the SCS group and the reduction in SINE (B1) expression in XY^{sry} mice as well as enriched H3K9me3 marks suggests that SINE elements may directly recruit heterochromatin in mouse thymus and thereby inducing the silencing of nearby genes. Furthermore, high levels of proteasome binding on SINE elements [Figure 3.2] could have potential implications regarding to proteasome-dependent regulation of SCS genes.

Long repeats such as L1 are known to facilitate the spreading of heterochromatin (Speek, 2001; Whitelaw and Martin, 2001) or they can disrupt the proper regulation of nearby genes by inducing antisense transcripts (Tchenio *et al.*, 2000). Although LINE elements were found to be less enriched in SCS set compared to a control group [Figure 4.8A], their expression seems to be enhanced in the presence of *Sry* in the XY background [Figure 4.8E]. This could be due to the fact that LINE elements contain binding sites for SRY and SOX proteins (Nigumann *et al.*, 2002). Indeed, many human genes were found to be transcribed from the antisense promoter of the L1 (Atharikar *et al.*, 2004; Yang *et al.*, 2003). Based on the fact that L1 is expressed more in the presence of *Sry* in the XY background, it is possible that *Sry* may interfere with the regulation of SCS genes through inducing L1 transcription and

silence nearby genes possibly by promoting antisense transcription. In addition to Sry, direct binding of the transcriptional regulators YY1 and RUNX3 were also reported for mammalian L1 promoters based on *in vitro* assays (Athaniyar *et al.*, 2004; Yang *et al.*, 2003). Therefore their contribution in the sex chromosome complement sensitivity phenomenon could be also considered.

4.3.5 Summary and future directions

Understanding how sexual dimorphisms operate and are regulated, will substantially enhance our knowledge about gender development and open new avenues for the treatment of gender-biased diseases such as several autoimmune and neurological diseases as well as cancer. In this chapter, it was questioned whether differences in gene regulation mechanisms directly participate in the establishment of this phenomenon and if so, whether they are affected by sex chromosome complements. According to previous literature and findings here, it is likely that such effects take place as a combination of various factors affected by Sry and sex chromosome complement. Although some of them were addressed in this thesis, many others remain to be investigated:

‘Sink’ effect: The presence of an inactivated X chromosome can titrate away heterochromatin factors from autosomal targets. Here, it was shown that heterochromatic silencing of hCD2 is relieved based on the dosage of X chromosomes. To test this hypothesis further; the *Xist* transgene, which is the main player of inactivation, can be placed on an autosome and heterochromatin silencing can be tested using the hCD2 transgene or other heterochromatic genes. Perhaps, addition of heterochromatic chromosomes from other species could also be considered.

A direct transcriptional effect of Sry: Here it was shown that Sry has a repressing effect on hundreds of sex chromosome complement sensitive genes as detected by microarrays on mouse thymus. This repressing effect was thought to compensate such genes, which would otherwise be over-expressed in an XY background. Still, whether effects of Sry is indeed direct, needs to be addressed after elimination of sex hormones via gonadectomy. In order to detect the presence of Sry in adult tissues, proper antibodies should be developed. If the antibody problem cannot be overcome, a mass spectrometry approach could be considered. A genome-wide Sry ChIP approach will give very useful information regarding to direct Sry effects. For this, transgenic mice with myc-tagged Sry can be considered (Sekido and Lovell-Badge, 2008).

HP1 as a key player: Based on previous literature, it was thought that the repression by Sry can be mediated by the interaction of Sry with KRAB domain proteins, which in turn interact with TIF1 β that recruits HP1. Importantly, this study showed that genes that are sensitive to HP1 dosage are indeed enriched within the sex chromosome complement-sensitive (SCS) genes. Interestingly, these SCS genes responded to the over-expression of HP1 β differently based on sex chromosome complements. Intriguingly, they were upregulated in XY females as opposed to the three other sex chromosome backgrounds of the four core genotype cross. Whether such an effect takes place via different enrichment levels of H3K9me patterns or HP1 binding, remains to be investigated via ChIP on SCS genes. In order to address a possible functional difference of HP1, its post-translational modifications should be investigated in four core genotype mice.

Sex chromosome-linked genes: Sry seems to exert its repressive function on SCS genes in a manner that is dependent on the Y chromosome complement, although it does not need a Y chromosome to give rise to a male phenotype. This suggests that factors on the Y chromosome act in a synergy with Sry or it is also possible that factors on the X chromosome counteract its effects. Therefore, both Y- or X-linked factors should be evaluated in terms of sexual dimorphisms paying particular attention to genes that escape XCI and Y genes which are not homologues of any X-linked gene. Although they are evolutionarily homologous, the histone demethylases *Utx* and *Uty* are known to exhibit functional divergence. Likewise, as the histone demethylases Jarid1c (X) and Jarid1d (Y) are subject to unbalanced expression levels it would be interesting to investigate their role using ChIP against these factors on the SCS genes.

Repetitive DNA: Here, it was shown that SINE elements are enriched within the SCS gene loci whereas LINE elements are impoverished; based on RepeatMasker analysis. Interestingly, SINE transcription is repressed just like major pericentromeric repeats in the XY^{sry} males, where LINE transcription seems to be enhanced. Considering the fact that SINE elements are rich in H3K9me₃ and proteasome as opposed to LINEs in mouse thymus, it is possible that SINEs serve to recruit heterochromatin and silence nearby genes, possibly in a proteasome-dependent manner. On the other hand, LINE elements, which have Sry binding sites, might interfere with nearby gene expression by inducing anti-sense transcription. Analysing the PEV of hCD2 transgene integrated with transposon sequences on the FCG model would give valuable information regarding to their sexually dimorphic behaviour.

PEV-like effects: It should also be underlined that sexually dimorphic effects on gene expression could be a result of PEV-like gene silencing, as exemplified by *Kremen1*

expression in this thesis. Many more genes could be subject to this phenomenon and they need to be investigated. Importantly, given that PEV is a metastable state and might be dependent on cell cycle variegation might only be detectable at the RNA level given that proteins are generally more stable than RNA. Therefore RNA FISH or single-cell based PCR applications should be considered.

Crucially, experiments with four-core genotype can give important clues about sex chromosome complement effects; however, this system lacks the tool to prove whether such effects take place via the presence of a Y chromosome or the absence of a second X chromosome. Therefore, experiments with XO mice should be considered. Moreover, in order to elucidate any effects that are carried via imprinting, it would be beneficial to take advantage from X^mO and X^pO mice.

4.4 Contributions

The original observations of heterochromatin influences on sexual dimorphism belong to Nicky Harker and Alexander Saveliev. Patrick Wijchers did all hCD2 FACS experiments and identified the 369 SCS genes by microarray analyses with the help of the MRC CSC Genomics Centre. Cihangir Yandim performed the validation of microarrays by Q-RT-PCR and also did the gene ontology and RepeatMasker analyses and is responsible for all the other results presented in this chapter. Patrick Wijchers and Richard Festenstein provided useful ideas for the discussion.

Mice in this study were kindly provided by Paul Burgoyne (MRC NIMR-London) and genotyping of four-core genotype mice were mostly performed by his staff.

CHAPTER 5
CONCLUSION

As with so many molecular aspects of a eukaryotic cell, the mechanisms that underpin the regulation of chromatin are indeed highly complex. The status of chromatin is determined by many factors including DNA sequences, chromatin modifiers and RNA interference [as shown in figure 1.12]. Heterochromatin, which is the highly compact state of chromatin, is mostly associated with repetitive DNA and has a tendency to spread, affecting the expression of nearby genes [as explained in section 1.5.3]. Although heterochromatin is traditionally thought to silence transcription by restricting the physical access of RNA polymerases and transcriptional activators, results both in this thesis and by others (review- Eymery *et al.*, 2009; Martens *et al.*, 2005; Ting *et al.*, 2011) revealed a basal level of transcription by RNAPII even at highly heterochromatic regions. This suggests that high chromatin compactness is not always enough to restrict the access of RNAPII and transcription factors in heterochromatic gene silencing. Moreover, factors normally associated with heterochromatin (e.g. H3K9me3 or HP1) are also found within the actively transcribed genes (Barski *et al.*, 2007; Mikkelsen *et al.*, 2007; Vakoc *et al.*, 2006; Wang *et al.*, 2008), implying that heterochromatin linked mechanisms may have a rate-limiting effect on the on-going transcription as well. Therefore, components of the heterochromatin machinery could have even broader impacts on RNAPII transcription than previously thought and affect gene regulation in multiple ways that do not only depend on chromatin compaction.

In order to understand how heterochromatin is regulated and the effects it introduces to the regulation of RNAPII transcription, this Ph.D. study focused on the pathologically silenced *FXN* locus. Here, heterochromatin was shown to be nucleated by the expanded (GAA)_n repeats and this could be relieved by the histone deacetylase inhibitor nicotinamide, which successfully upregulated the silenced *FXN* gene to potentially therapeutic levels. Interestingly, heterochromatin on the silenced *FXN* locus did not seem to affect the recruitment of RNAPII. Instead, it was found that elongating RNAPII gets stalled within the first exon and is degraded by the proteasome in a manner analogous to what takes place during transcriptional coupled DNA repair, but only in the presence of pathologically expanded (GAA)_n repeats. This was linked to the finding of enriched proteasome binding at the pathologically silenced *FXN* locus, in a pattern reminiscent of heterochromatin marks. In line with these observations, RNAPII levels were restored upon proteasome inhibition and *FXN* expression levels could be restored.

These results regarding to *FXN* gene regulation suggested a novel dimension for heterochromatin function. Accordingly, an independent set of sequences including heterochromatic repeat elements (i.e. pericentromeric repeats and SINE elements) as well

as inactive genes were also shown to be enriched in proteasome binding. In addition to the classic explanation of chromatin compaction prohibiting access by the transcription machinery to genes, this suggests that heterochromatin possibly induces gene silencing also by degrading RNAPII. It is now essential to investigate this further using genome-wide ChIP studies perhaps combined with studying the genome-wide effects of proteasome inhibition. Also, it will be interesting to study further the mechanistic link between the proteasome and heterochromatin.

Another interesting phenomenon explored in this thesis was the effect of heterochromatin on sexually dimorphic gene expression. Although hormones have long been thought to be the main responsible factor in sexual dimorphisms, results presented in this thesis suggest that these could in part be due to gender differences in heterochromatic gene silencing triggered by the difference in sex chromosome complement. Here, such effects were not only revealed by the mammalian hCD2 PEV system but also on hundreds of endogenous mouse genes. Interestingly, the effect of HP1 β was shown to depend on both sex chromosome complement and Sry. Notably, SINE elements were found here to be enriched in a gene group which showed sensitivity to sex chromosome complement. They were also more likely to be found nearby HP1 β -repressed genes [personal communication Patrick Wijchers and Richard Festenstein (MRC CSC, London)], and found here to have high levels of heterochromatin marks and proteasome binding [Figure 3.2]. Taken together, it is tempting to speculate that this repetitive element may be playing a role in the recruitment of heterochromatin and might participate in establishing a sex bias in gene expression by rendering genes sensitive to sex chromosome complement effects. Might this also suggest a link between the proteasome-mediated heterochromatic gene silencing and sexual dimorphism? This is a hypothesis that needs to be tested further possibly by performing proteasome ChIP on sex chromosome complement-sensitive genes. There are also many other questions that remain to be answered: Does the inactivated X chromosome act like a sink for heterochromatic factors? Does Sry have a direct transcriptional role on sexually dimorphic genes in adults? Are there any sexually dimorphic factors that influence the function of HP1? How do transposable elements affect this phenomenon? To what extent is the PEV-like gene silencing a general phenomenon that affects endogenous genes or are most genes insensitive to these effects?

Although a large number of questions remain to be answered, experiments presented here opened new avenues about how heterochromatin may function in gene regulation and how it could be influenced by various factors such as the proteasome and sex chromosome complement. The findings in relation to *FXN* gene have therapeutic implications for the

neurodegenerative disorder Friedreich's ataxia. Nicotinamide, which is a bioavailable sirtuin inhibitor in clinical use (review-Knip *et al.*, 2000), has already been approved for a proof-of-concept clinical trial on FRDA patients. The proteasome inhibitor PS341, which is also used in the clinic to treat multiple myeloma (review-Richardson *et al.*, 2006), also showed a potentially therapeutic effect on *FXN* expression. One might therefore consider a 'cocktail therapy' with both of these drugs to increase the expression of *FXN* in FRDA whilst keeping the toxic effects to a minimum. Also, other specific sirtuin inhibitors such as splitomicin and salermide are of potential interest for the treatment of FRDA. Figure 1.23 presented a large number of diseases that are caused by abnormal repeat expansions. Considering the fact that repetitive DNA is likely to halt transcription and induce heterochromatinisation in conditions such as Fragile-X syndrome, it is possible that sirtuin inhibitors may have a potential effect on these diseases as well. Also, sirtuin knockout mouse models would be beneficial in terms of assessing the effects of sirtuins on the expression of specific genes and heterochromatic repeats.

Finally, findings in relation to sexually dimorphic gene expression provided potentially important information for understanding the nature of sex-biased diseases revealing a previously unrecognised level at which the differences between the sexes might be determined. Although the factors which are involved in maintaining the sex chromosome and hormone balance have not been narrowed down yet, results presented in this thesis and previously published studies in relation to X and Y specific factors revealed a number of potential candidates that could establish this phenomenon. Understanding their role and mechanism would give invaluable clues about sex-biased diseases including certain types of cancers and neurodegenerative as well as immunological conditions. Therefore, more rational therapies against such diseases could be developed. This Ph.D. study focused on mouse thymus; however, brain and liver are other characteristic tissues known to be subject to sexual dimorphism. Therefore studies presented here are worth being repeated on these critical tissues.

CHAPTER 6

MATERIALS AND METHODS

6.1 General techniques and storage conditions

Distilled water was used to prepare all stock solutions and reactions. All reagents were purchased from Sigma and all solutions and reactions were made up in water unless otherwise stated. All small volume centrifugation steps were carried out at room temperature using a desktop centrifuge (Eppendorf) and at 4°C in a refrigerated centrifuge (Eppendorf). A refrigerated centrifuge (Heraeus 1.0R) was used for the falcon tubes. DNA containing solutions were stored at -20°C in the short term and at -80°C in the long term. Solutions containing RNA were generally snap frozen and stored at -80°C. Mouse tissues were stored at -80°C. All antibodies were stored in the short term at 4°C and long term at -20°C (unless otherwise stated by the manufacturer's guidelines) - if necessary in the dark. Oligonucleotide primers were obtained from Sigma, and stock primer solutions (100 µM) and aliquots (10 µM) were stored at -20°C.

6.2 Preparation of drug treatments and storage conditions

All drugs were prepared under sterile conditions under a cell culture-hood. Nicotinamide was purchased from Sigma (cat#72345) and stored in room temperature. 1M stock solution was prepared using the appropriate culture medium for cells or physiological saline for animals. Before injection to animals, nicotinamide solution was filtered using a 0.2µm syringe filter (VWR). PS341 (Velcade®, 3020µM) was kindly provided in a sterile bottle (in physiological saline) by Hammersmith Hospital (London) pharmacy. The bottle was stored at -20°C until used. For cell culture, it was used directly from the 3020µM stock, for animal treatments it was diluted 10x in sterile physiological saline. MG132 was purchased from Sigma and dissolved in DMSO to prepare a 10mM stock solution. Alpha-amanitin and DRB were also purchased from Sigma. Alpha-amanitin was dissolved as 1mg/ml in culture medium whereas DRB was dissolved in DMSO to prepare a 10mM stock solution. Please see the main text for the final drug concentrations.

6.3 Cell culture and isolation of primary lymphocytes

EBV-transformed lymphoblastoid cell lines were obtained from the National Institute of General Medical Sciences (NIGMS) Human Genetic Cell Repository at the Coriell Institute, Camden, New Jersey, USA. Lymphoblast cell lines were grown in RPMI 1640 medium with 2 mM L-glutamine and 10% fetal calf serum at 37 °C in 5%CO₂. The culture medium was replaced every 2-3 days depending on the growth rate of the cells. The cell number was maintained between 0.5-1.2 x 10⁶ cells during culture. Lymphoblastoid cells were not cultured more than 6 weeks and fresh stock from the -80°C was used to replace them after this time. Primary human lymphocytes were extracted from the blood

samples taken from FRDA patients and healthy individuals (full ethical approval had been obtained from the local ethics committees at the National Hospital for Neurology and Neurosurgery and the Hammersmith Hospital, London) using the Ficoll-Paque™ PLUS (GE Healthcare). The blood samples were dispensed into multiple 50ml falcon tubes (15 ml each). Next, sterile pre-warmed PBS was added to make up a total volume of 20ml. About 13ml of Ficoll was added to top up the blood/PBS suspension and the samples were centrifuged at 1500rpm for 15min at room temperature. The interface buffy-coat containing lymphocytes was transferred into fresh 50ml falcon tubes, diluted 2 fold with sterile PBS and centrifuged at 1500rpm for 15min at room temperature. The supernatant was discarded and pellet was resuspended and cultured in the same culture medium as lymphoblastoid cells (stated above). Usually, ~1.5 million lymphocytes were obtained per 1ml blood.

Mouse thymocytes were obtained by teasing the thymus tissue apart and suspending the cells in PBS. Next, the cells in solution were filtered using the BD cell strainer (70µm). An intact thymus from a 4 weeks old female would give ~100 million thymocytes. These cells were cultured in RPMI 1640 medium with 2 mM L-glutamine, 10% fetal calf serum and 50µM 2-mercaptoethanol.

For any drug treatment that takes longer than 24 hours, cell culture medium was changed and replaced with new drug every day. All cells were centrifuged at 1200 rpm for 5 minutes, when needed.

6.4 Experimental mice and genotyping

All necessary approvals for any procedure applied here were obtained officially from the U.K. Home Office. For genotyping of animals, ear punches were lysed in 0.1M Tris HCl (pH 8.0) + 5mM EDTA (pH 8.0)+0.2M NaCl+0.2 %SDS+ proteinase K (Roche, 200 µg/ml, freshly added each time) overnight in a 55°C shaker. Next day, proteinase K was deactivated by incubating the samples at 95°C for 15 minutes and afterwards the samples were centrifuged briefly at 4°C. The lysed material was diluted 4 fold in PCR-grade water and used directly for PCR. For the PCR reaction the following mastermix was prepared per one reaction using the Biotline Biotaq® PCR kit:

10x NH4 reaction buffer	2.5µl
MgCl2 (50mM)	1.25 µl
BSA (100x)	0.75 µl
dNTPs (10mM mix)	0.5 µl
Primers	See below*
Biotaq DNA polymerase	0.2 µl
ddH2O	Up to 25 µl
DNA	2.0 µl
TOTAL	25µl

*all primers from 100µM stock solution. See supplementary table S.6 for primer sequences.

YG8 mice genotyping: 0.5µl hFXN3_L + 0.5µl hFXN3_R + 0.25µl FXN-KO_WJ5 +0.20µl FXN-wt-WN39 + 0.05µl FXN-KO_WC76 primers. On gel, wild type mouse *Fxn* is the upper band (~480bp), human *FXN* is the middle band (~300bp) and wild type *Fxn* knockout is the lower band (~250bp).

HP1γ deficient mice genotyping: 0.5µl common_L+ 0.5µl mutant_L +0.5µl wild type_R primers. Wild type band is 501bp whereas mutant band is 525bp.

HP1β over-expressing mice genotyping: 0.5µl from each M31 primers. The transgenic animals give a 1.3kb band whereas there should be no band for wild type animals.

Four core genotype mouse genotyping: 0.5µl from each YMT primers. These give a large band specific to the Y chromosome long arm. If the band is present in a female, then genotype is XY⁻, if the band is present in a male, the genotype is XY⁻sry. If not present in female, the genotype is XX. If not present in male, the genotype is XXsry.

hCD2 mouse genotyping: 0.5µl from each hCD2 primers. 651bp band for the hCD2 transgene.

A touch down PCR programme was used for all genotyping purposes:

1. 95 for 2:00
2. 95 for 0:30
3. 65 for 1:30
-0.5 per cycle
4. 70 for 1:30
5. Goto 2 for 20 times
6. 94 for 0:30
7. 55 for 1:30
8. 70 for 1:30
9. Goto 6 for 20 times
10. 70 for 7:00
11. 10 for ever
12. End

6.5 Phenol-chloroform extraction of DNA

Proteins were removed from aqueous solutions of DNA by extraction with equal volumes of phenol: chloroform: isoamylalcohol (25: 24: 1). The samples were centrifuged for 14 minutes at 12 000 g at 4°C to separate the phases. The aqueous upper phase was transferred into a new tube. The DNA in the aqueous phase was precipitated by mixing with 3 M of sodium acetate (NaOAc) (1:10 of the original DNA solution volume) and precipitated by mixing it with two volumes of 100 % ethanol. The samples were incubated at -80°C for 1 hour. Next, they were centrifuged for 30 minutes at 12 000 g at 4°C and the supernatant was discarded. Subsequently 70 % ethanol was added and centrifuged for 15 minutes, at 12 000 g at 4°C. The pellet was air dried and re-suspended in DEPC treated water. DNA concentration was measured using NanoDrop Spectrophotometer ND-1000 with ND-1000 v3.3.1 software. As the fraction of the absorbed UV radiation is directly proportional to the concentration of DNA, an optical density value (OD_{260}) of 1 equals a concentration of 50 µg/ml. The purity of the solution is given by the ratio of A260/280. This ratio should be around 1.8.

6.6 Gel electrophoresis of DNA

Agarose gel electrophoresis was used to separate 50 bp - 10 kb DNA fragments. Electrophoresis was carried out using a horizontal gel electrophoresis apparatus (Gibco BRL), connected to a power supply (Biorad Powerpac 300). As the electrophoresis 1X TBE buffer was used (90mM Trisborate and 2mM EDTA). Agarose gel was made from 1.5 % pure agarose (Invitrogen) melted in 1X TBE buffer. After the agarose was cooled down to ~60°C, 0.5 µg/ml of ethidium bromide (Sigma, UK) solution was added and mixed well in a fume hood. The agarose was transferred to a horizontal gel tray and wells were formed with appropriate combs. The DNA solutions were re-suspended in 6X MassRuler DNA loading dye (Fermentas). The samples were loaded into the wells of the gel as well as DNA size marker (MassRuler DNA Ladder, Fermentas). Electrophoresis was carried out at 80 Volts. DNA was visualised with a GelDoc 2000 (Biorad) system using Quantity One software.

6.7 Total RNA and protein extraction using Trizol®

Total RNA from EBV-cell lines, human primary lymphocytes, thymocytes and mouse tissue samples was isolated using the Trizol reagent (Invitrogen). For the RNA extraction from the suspension cells, a pellet of $\sim 5 \times 10^6$ cells were homogenised by pipetting up and down harshly in 1ml Trizol. For tissue samples, frozen tissue samples were homogenised using the IKA ultra-turrax T25 homogeniser at maximum power in 1ml Trizol (bubbles were removed afterwards). Next, homogenised cell or tissue samples were incubated for 5min at room temperature. Afterwards, 200µl chloroform per 1ml Trizol was added and mixed by

15sec vigorous shaking followed by a room temperature incubation for 2-3min. Samples were then centrifuged at 12000g, 4°C for 15min. Aqueous phase was transferred to a fresh tube and organic phase was saved for protein isolation (organic phase kept on ice). Next, 500µl of isopropanol per 1ml of Trizol was added to the retained aqueous phase and mixed by vortexing. This was followed by another room temperature incubation for 10min. Samples were then centrifuged at 12000g, 4°C for 15min. Supernatant was removed and 1ml 70% ethanol per 1ml of Trizol was added. Samples were mixed by vortexing and centrifuged at 7500g, 4°C for 15min. Finally, supernatant was removed and RNA pellets were air-dried briefly. In order to eliminate any possible DNA contamination, freshly-isolated RNA underwent DNase treatment with DNA free kit (Ambion). This was performed by adding 40µl master mix containing 33µl RNase free water, 2µl Superase, 4µl 10X DNaseI buffer and 1µl DNaseI. RNA and reaction mixtures were incubated 10min at 37°C. 4µl of inactivation reagent was then gently mixed with each sample, which was subsequently incubated at room temperature for 2min. This was followed by centrifugation at 10000g for 2min. 35µl supernatant which contained RNA isolated was retained for cDNA synthesis. The quality and yield of RNA was measured by the Agilent Bioanalyser RNA nano-chip complying the manufacturer's guidelines. RNA was stored in -80°C.

For protein isolation, the stored organic phase (pink) was added with 0.3 ml 100% ethanol per 1 ml of Trizol used for the initial homogenization. Samples were mixed by vigorous shaking. Next, the samples were incubated at room temperature for 2-3 min and any DNA content was sedimented by centrifugation at no more than 2000xg for 15 minutes at 2°C to 8°C. The phenol-ethanol supernatant was then transferred into a fresh 2 ml eppendorf tube and added with 1.2ml isopropanol followed by a 10 minute incubation at room temperature. The samples were then centrifuged at 12000g for 10 minutes at 4°C. The supernatant was aspirated and the protein pellet was washed three times with 0.3M guanidine hydrochloride solution (each wash 20 minutes room temperature incubation followed by 8000g centrifuge for 1 min. at 4°C). Next, the protein pellet was washed once with 2ml absolute ethanol. If desired, the protein pellet was stored in ethanol for a month at 4°C or for a year at -20°C. Finally, ethanol was aspirated and the pellet air dried. The pellet was then dissolved in 0.5ml 1% SDS for general purposes. If the protein sample would undergo Mitosciences dipstick assay, it was dissolved in 0.5ml Buffer A from the Mitosciences Kit. For enhancing the dissolving process, protein samples were shaken at 55°C for half an hour. Protein solutions were stored in -80°C.

6.8 Complementary DNA (cDNA) synthesis

Total RNA which was of high quality [RNA integrity factors ≥ 8.5 by the Agilent Bioanalyser, see supplementary figure S.1A] was reverse transcribed into complementary DNA with ThermoScript[®] kit (Invitrogen). For each reaction, 50-250ng of random hexamer (for primary transcript detection) or oligoDT (for mRNA detection) and 2 μ l of 10mM dNTP mix was mixed with 1.25 μ g of RNA and sterile water was then added to make up a total volume of 13 μ l. The reaction mixture was then incubated at 65°C and then placed on ice. 4 μ l of 5X cDNA synthesis buffer, 1 μ l of 0.1M DTT, 1 μ l RNaseOUT[®] and 1 μ l of ThermoScript[®] reverse transcriptase (15U/ μ l) was mixed with the hexamer/oligoDT+RNA+primer mix. Next, samples were incubated at 50°C for 60min and then at 85°C for 5 minutes. Samples were stored in -20°C.

6.9 Chromatin immunoprecipitation (ChIP)

Chromatin immunoprecipitation (ChIP) was carried out following the “*Protocol for the fast chromatin immunoprecipitation (ChIP) method*” published in Nature Protocols (Nelson *et al.*, 2006). All the related solutions can be found in section 6.18.

For suspension cells; $\sim 1.5 \times 10^7$ cells in 40ml RPMI medium were cross-linked by incubating with 1.6ml of 37% w/v formaldehyde (final concentration 1.42%) for 15min. 141 μ l of 1M glycine per 1ml culturing medium (final concentration of glycine 125mM) was added to quench the formaldehyde reaction and incubated for 5min. Samples were then centrifuged at 2000g for 5min at 4°C and washed twice with ice-cold PBS before the lysis.

In tissue ChIP, tissue samples from -80°C storage were put in a grinding bowl (mortar) that contains liquid nitrogen. Tissue sample was then squashed (with a pestle) in liquid nitrogen into small pieces and transferred into 10-20ml room temperature PBS with 37% w/v formaldehyde (final concentration of formaldehyde 1.42%) and incubated for 10min at room temperature accompanied by gentle mixing. 141 μ l 1M glycine per 1ml PBS was added to quench the crosslinking reaction and then mixed gently for 5min. Samples were then centrifuged at 2000g, 4°C for 5min. Supernatant was discarded and the tissue pellets were washed twice with ice-cold PBS. After the last wash, 2ml of cold PBS was added to each sample and then the samples were homogenised with an IKA ultra-turrax T25 homogeniser at maximum power. Homogenised samples were subsequently centrifuged at 2000g, 4°C for 5min and the supernatant was discarded.

Both for suspension cells and tissue samples, lysis was performed by mixing the samples vigorously with 1ml IP buffer, which was added with the protease inhibitor cocktail (Sigma)

and PMSF (see section 6.18). They were centrifuged at 12000g for 2 min. and washed once with IP buffer containing protease inhibitors and PMSF (IP++). The nuclear pellet was then resuspended in 1ml IP(++) buffer and sheared by sonication (Bioruptor, Diagenode) for 1.5 hour, high energy (with interval of 0.4min on, 0.5 min off). Following sonication, samples were centrifuged at 12000g, 4°C for 10min. Sheared chromatin in supernatant was retained. 30µl of chromatin was loaded on a 1.5% agarose gel and the sonication efficiency was visualised. A well-sonicated material typically gives a DNA smear between 200-500bp (see supplementary figure S.3A for a representative sonication gel picture.)

Specific amounts of different antibodies (see supplementary table S.2) were added to 200µl aliquot of sheared chromatin obtained respectively for each ChIP. Always, one 200µl aliquot did not receive any antibody in order to check the background levels. For input DNA, a 20-50µl input material was stored in -20°C at this point, for later purification (see below). Binding of antibodies to their targets was enhanced by incubating the samples in a Bioruptor (Diagenode) for 30 min, low energy (with interval of 0.4min on, 0.5 min off). Each sample was then centrifuged at 12000g, 4°C for 10min and 180µl of sonicated material was obtained. Antibody-bound complexes were immunoprecipitated with 50µl Millipore protein-G IgG agarose beads [pre-washed with IP buffer and 2x diluted in IP buffer] for the Friedreich's ataxia project (chapter 2) and undiluted dynabeads® Protein G (Invitrogen) for the proteasome project (chapter 3). Pre-washed and pre-diluted (2x) IgM beads (Sigma, #A4540) were used for the RNAPII-S2p antibody. The samples were then rotated at 4°C for 12 hours at least.

Beads with antibody-bound complexes were washed 6 times with IP buffer (without protease inhibitors and PMSF) after the rotation period. Immunoprecipitated protein/DNA complexes were de-crosslinked and the ChIP DNA was released using 110µl Chelex -100 (10% w/v) (BIO-RAD) per sample. Beads with antibody-bound complexes and Chelex -100 were gently mixed and then heated twice at 95°C for 7min with brief vortex in between. Samples were then centrifuged at 12000g, 4°C for 2min and 80µl of supernatant was retained. 200µl of sterile water was added to the beads with Chelex -100 and the solution was centrifuged at 12000g, 4°C for 2min. 200µl of supernatant was obtained to make up a 280µl ChIP DNA sample in total. The pre-stored input DNA could be recovered by precipitating the DNA with 600µl absolute ethanol and centrifuging at 12000g at 4°C for 15 minutes. The precipitated DNA was then washed with 70% ethanol once and centrifuged at 12000g, 4°C for 5 min. The resultant input DNA pellet was then treated with Chelex resin in the same way with ChIP samples. The ChIP and input DNA was stored in -20°C.

6.10 RNA immunoprecipitation (RIP)

RIP was performed by modification of the ChIP protocol. As an extra point, the IP buffer contained RNase inhibitor (RNasin 60 Units/ml, Promega). After the shearing sonication, the extract was treated with DNase I (30 Units/ml, Applied Biosystems) in 25mM MgCl₂, 5mM CaCl₂ and RNasin for 25 min at 37°C. Nuclease digestion was stopped by adding EDTA at a final concentration of 20mM. Immunoprecipitation was performed as described above for ChIP. However, the rotation took place only for 3 hours. All subsequent steps were in concordance with the ChIP protocol. Finally, instead of Chelex, the elution was performed by adding 75 µl elution buffer [50 mM Tris-HCl pH8.0, 10 mM EDTA, 1% SDS, RNasein 60U/ml] and incubating for 3 hours at 65°C, shaking very gently. Next, the SDS was diluted by adding 200µl of PCR grade water. Finally proteinase K (Roche, final conc. 200µg/ml) was added and the samples were incubated for 2 hours at 55°C, shaking gently. After this final step, they underwent RNA extraction as described before (see section 6.7).

6.11 Quantitative real-time polymerase chain reaction (Q-RT-PCR) for RNA expression and ChIP analysis

Q-RT-PCR was performed using the SYBR® Green Jumpstart™ Taq ReadyMix. Before preparing the PCR setup, cDNA material was pre-diluted 2.5x in PCR-grade water whereas the ChIP DNA was used directly without further dilution. The reaction mixture was prepared in low-profile 96-well white PCR plates (BIORAD). A single Q-RT-PCR reaction contained 10µl of SYBR® Green Jumpstart™ Taq ReadyMix (Sigma), 0.5µl of 10µM forward primer, 0.5µl of 10µM reverse primer (see supplementary tables S.3, S.4 and S.5) and 5µl DNA sample. The PCR-grade DEPC treated water was added to make up a total volume of 20µl. cDNA samples were prepared in triplicate and ChIP samples in duplicate. The PCR reaction was performed and monitored using the Chromo4 DNA engine (MRJ) with Opticon Monitor 3 (BioRad) software. The following PCR programmes were used for specific purposes:

For RNA expression analysis:

1. Incubate at 95°C for 2 minutes //enzyme activation
2. Incubate at 95°C for 30 seconds
3. Incubate at 58°C for 30 seconds
4. Incubate at 72°C for 30 seconds
5. Plate Read
6. Incubate at 80°C for 1 second
7. Plate Read
8. Incubate at 82°C for 1 second
9. Plate Read
10. Incubate at 85°C for 1 second
11. Plate Read
12. Goto Line 2 for 40 times

13. Melting Curve from 70°C to 96°C, read every 0.5°C – hold 1second
14. END

For ChIP analysis:

1. Incubate at 95°C for 10 minutes. // enzyme activation
2. Incubate at 95°C for 30 seconds
3. Incubate at 58°C for 30 seconds
4. Incubate at 72°C for 30 seconds
5. Plate Read
6. Incubate at 80°C for 1 second
7. Plate Read
8. Incubate at 82°C for 1 second
9. Plate Read
10. Incubate at 85°C for 1 second
11. Plate Read
12. Go to Line 2 for 40 more times
13. Melting Curve from 70°C to 96°C, read every 0.5°C – hold 1second

After the reaction was complete, the threshold was arbitrarily placed (on the linear part in the logarithmic scale) and the C(t) values were imported from Opticon Monitor 3 to MS excel software after checking the melting curves. For each primer, a primer efficiency factor was calculated using a series of DNA dilutions. Next, the quantitation was made using the Pfaffl method taking the efficiencies into account [as previously described (Pfaffl, 2001)]. The calculations were made on MS Excel using the average C(t) values obtained from technical replicates, based on the following formula:

Efficiency factor : $E = 10^{-1/\text{slope}}$

*slope is calculated by the tangent of the dilution curve (DNA amount in log10 versus Ct)

Expression ratio : $(E_{\text{target}})^{\Delta C_{t \text{ target (control-treated)}}} / (E_{\text{ref}})^{\Delta C_{t \text{ ref (control-treated)}}}$

For all RNA expression experiments, the amount of amplified cDNA was normalised against the values obtained from a housekeeping gene (e.g. *β-actin*). In terms of ChIP, the value obtained from the no-antibody control was subtracted from the signal obtained by the immunoprecipitated material. For the Friedreich's ataxia project (chapter 2), ChIP loading/nucleosome density differences were normalised as the amount of ChIP DNA was calculated as a percentage of the signal obtained from the histone H3 signal. Still, control genes (see supplementary figure S.4) also served as a secondary check for ChIP and PCR efficiency. For the proteasome project (chapter 3), the ChIP signal was calculated as a percentage of input DNA. The C(t) values obtained from the input material were mostly within the same range for each specific primer set [the difference below 0.3 C(t)] between experimental replicates, indicating that the ChIP loading levels were not significantly different.

6.12 Frataxin protein measurement using the Mitosciences dipstick assay kit

The dipstick kit [lateral flow immunosandwich assay] was purchased from Mitosciences, Oregon U.S.A. (cat# MSF31). The concentration of total protein samples were first measured by the bicinchoninic acid assay kit (BCA kit) from Sigma. A working BCA solution was prepared by mixing 1ml of reagent A with 20 μ l of reagent B per sample. 50 μ l of the protein sample was added to the working solution and then the samples were transferred into 10mm spectrophotometer cuvettes (Fisher). Next, they were incubated at 37°C for half an hour. The coloured reaction was measured as the absorbance at 562nm by the Ultraspec 2100 Pro spectrophotometer (GE Healthcare Life Sciences). A standard curve was calculated using a bovine serum albumin solution. An example is given on supplementary figure S.2A. The protein concentration was calculated by interpolating the measured absorbance on the BCA standard curve. Next, the original protein samples were carefully diluted in buffer A to obtain an exact final concentration of 0.2 μ g/ μ l each. Next, 25 μ l of the diluted samples were added to the plate wells (provided) which contain the gold-conjugated FXN antibody. 25 μ l of buffer B was then added on the surface and the solution was mixed by pipetting up and down, in order to totally dissolve the coloured antibody and mix it with the protein solution. The dipsticks, which contain an immobilised FXN antibody, were then gently placed onto the well and they were let to soak in the material at room temperature for half an hour. The existence of the reaction was checked by the appearance of the upper band, which is non-specific. The FXN signal is the lower band. Finally, the dipsticks were carefully washed with 30 μ l buffer C by carefully letting the buffer run over the sticks. Dipsticks were then air dried at room temperature for an hour in a dark place. The signal was then measured by the Mitosciences MS1000[®] dipstick reader. A standard quantitation curve was drawn by using recombinant FXN [Mitosciences (cat# MSF42)] as shown in supplementary figure S.2B. The absolute measurements for the actual samples are calculated by interpolating the absorbance values from the standard curve by using an online polynomial root calculator [<http://xrjunque.nom.es/precis/polycalc.aspx>].

6.13 Western blotting

Proteins were resolved by running the total protein samples on a polyacrylamide gel. Here, the Bio-Rad 2-D system with 1mm plate separation was used. The main part of the gel (the resolving gel) was prepared as a 12% gel made up from acrylamide:bis-acrylamide 29:1, 4x resolving buffer (1.56M Tris pH8.8, 0.4%SDS) and dH₂O to which ammonium persulphate and TEMED (to final concentrations of 0.1% each) were added. After the gel was loaded and set, the upper part of the gel (stacking gel) was prepared as an 8% gel made up from acrylamide:bis-acrylamide 29:1, 4x stacking buffer (520mM Tris pH6.8, 0.4%SDS) and dH₂O

to which ammonium persulphate and TEMED (to final concentrations of 0.1% each) were added. This was loaded until the gel plates were full and the comb was inserted.

When fully set, the comb was removed, wells flushed and the acrylamide gel loaded into the electrophoresis tank which was filled with 1x running buffer (per litre of H₂O, Trizma base 3.02g, glycine 18.8g and SDS 1g). The protein samples were boiled with 2x loading (Laemmli) buffer (4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.004% bromophenol blue, 0.125 M Tris HCl pH6.8) for 5 minutes and then stored on ice before loading 15-30µg of protein per well. Protein concentrations were assayed against BSA dilution standards using Bio-Rad protein assay and the Ultraspec 2100 Pro spectrophotometer (GE Healthcare Life Sciences) and the respective manufacturer's recommended procedures. One lane was loaded with 3-5µl of Precision Plus Protein Kaleidoscope Standard (Bio-Rad). The gel was then run at 200 Volts.

When the electrophoresis was complete, an Immuno-Blot PVDF membrane (Bio-Rad #162-0177) of the same dimensions as the gel was prepared with successive washes in 100% methanol, dH₂O and finally in transfer buffer (per 1000mls, 2.9g glycine, 5.8g Trizma base, 0.37g SDS, 200mls methanol). A stack was created from a base of 3 pieces of 3mm Whatman paper (Whatman) soaked in transfer buffer, the prepared PVDF membrane, the gel, topped off by 3 more pieces of 3mm Whatman paper soaked in transfer buffer. The stack was then placed on the anode of a TE77 semi-dry transfer unit (GE Healthcare Life Sciences) and the cathode was closed on top of the stack. Transfer of protein from gel to membrane was then achieved by subjecting the stack to a current of 80mA (max. 30V) for 90 minutes. Transfer was confirmed by staining the membrane in Ponceau Red (per 500mls, 2.5g of Ponceau S (Sigma #3504) and 5mls glacial acetic acid) to reveal the protein bands. After washing with dH₂O to remove Ponceau Red and briefly with TBST (per 1000mls, NaCl 8.01g, Trizma base 1.21g, pH adjusted to 7.5 with HCl, 0.5mls Tween 20) the membrane was blocked for a minimum of 1 hour with TBST+ 5% milk on a Stuart Gyrotory Rocker SSL3. Next, the antibody was added at an appropriate dilution rate (see supplementary table S.2) and shaken on the rocking platform gently for 3 hours. The blot was then washed 4 times for 5 minutes each in TBST. Subsequently, the surface of the blot was covered with an HRP-conjugated secondary IgG antibody, see table 7.2) in TBST+5% milk and rocked as above for 30 minutes prior to washing as above.

The presence of HRP on the blot was then detected using ECL Plus Western Blotting Detection Reagents (GE Healthcare) using the manufacturer's recommended protocol. The image was then compared with the protein ladder on the original blot to estimate the size of protein detected.

6.14 Immunofluorescence (IF) staining

The antibodies were pre-labeled with Invitrogen microscale antibody labelling kit. Here, 40µg of antibody was pre-labeled using the resin columns provided, complying the manufacturer's guideline. The labelling was performed with Invitrogen Alexa Fluor® 488 and 594.

For the IF procedure, 0.2×10^6 cells in 100µl PBS were placed gently onto poly-lysine coated slides (Polysciences). After the cells settled down on the slide, the PBS was aspirated and the cells underwent fixation with 1% formaldehyde (in PBS) for 15 min. at room temperature. Subsequently, formaldehyde solution was removed with three consecutive PBS washes. Next, cells were permeabilised with pure methanol for 5 min. at room temperature. The fixed and permeabilised cells on slides were then washed with 2.5 % BSA in PBS (blocking solution). Subsequently, non-specific antibody binding sites were blocked by incubating the cells in the blocking solution for 30 minutes at room temperature. Next, cells on slides were incubated with 100µl of pre-diluted antibody/PBS solution (see supplementary table S.2) for 1 hour at 4°C in a dark place. Afterwards, the cells on slides were washed with PBS three times (each 5 minutes). Finally, slides were mounted with coverslips in Vectashield mounting medium containing DAPI (Vector Laboratories) and were examined under Leica Confocal Microscope (photographed with a photometrics Coolsnap HQ CCD camera).

6.15 Flow cytometry (FACS)

For thymocytes, the cells were fixed with 1% formaldehyde in PBS for 15 min. at room temperature in eppendorf tubes. If the protein of interest was cytoplasmic or nuclear, a permeabilisation step was applied using 0.05% w/v saponin in PBS, pipetting up and down gently and incubating at room temperature for 15 min. Next, cells were washed three times with staining buffer (0.5% w/v BSA in PBS). Each wash consisted of a 1 min. room temperature incubation followed by centrifugation at 1200rpm for 5 min.

For peripheral blood cells, red blood cells needed to be eliminated prior to FACS procedure. They were lysed at 37°C for 5 minutes by washing with Gey's solution made up fresh as 20:5:5:70 mixture of stock A:stock B:stock C:dH₂O. The composition of stock solutions is given in section 6.18. The cells were then centrifuged at 300g at 4°C in round bottom glass FACS tubes. The Gey's solution was subsequently removed by aspiration and cells were washed three times with staining buffer (see above).

After this point, specific antibodies at appropriate dilution rates (see supplementary table S.2) were added to cells in 50µl staining buffer. The cell suspension was mixed gently by pipetting up/down and incubated on ice for 1 hour in a dark place. Next, cells were washed

three times with staining buffer and finally re-suspended in 500µl staining buffer. They were then analysed by Beckton Dickinson FACScalibur using the CellQuest software (see supplementary figure S.10) after appropriate fluorescence compensation rates were applied.

6.16 Microarrays

Here, very high quality RNA samples (Agilent Bioanalyser RNA integrity score >9.0) were used. cRNA of three biological replicates for all samples was prepared from 10 µg total RNA using a one-cycle labeling protocol following Affymetrix instructions. Fragmentation of antisense cRNA and hybridisation to Affymetrix mouse genome 430 2.0 arrays was performed at the CSC/IC Microarray Centre according to the manufacturer's instructions. Further details are available from the CSC/IC Microarray Centre web site [<http://microarray.csc.mrc.ac.uk/>]. Raw hybridisation output files (.CEL) were imported into the R statistical analysis suite for downstream analysis. Pre-processing using the RMA bioconductor package was followed by the analysis of differentially expressed genes and the quality checks were done carefully (see supplementary figure S.11). Gene selections were exported to MS Excel spreadsheet software. Genes that showed fold-changes equal or larger than 1.2-fold with a $p < 0.05$ (Student's t-test) were considered differentially expressed (Shi *et al.*, 2008). Gene ontology and KEGG pathways were analysed using DAVID software. All Affymetrix data have been deposited in NCBI's Gene Expression Omnibus (Edgar *et al.*, 2002) and are accessible through GEO Series accession number GSE21822.

6.17 Statistics and softwares

In this thesis, two tailed and paired student's t-test was applied to assess whether the values from an equal number of samples from two different experimental group were significantly different. This was performed using the MS Excel software. All other statistical tests were performed using the NCSS statistical analysis software (Number cruncher statistical system, Kaysville, USA). To prove that the values in one group were higher in samples with equal sample size and variance, Mann-Whitney U test was applied. Kolmogorov-Smirnov test was applied for the comparison of non-parametric values from two groups, which do not necessarily exhibit normal statistical distribution patterns. To assess statistical difference in more than two groups, ANOVA test was applied. Fisher's exact test of over-representation was performed in order to assess the enrichment of events (genes) within a group as opposed to another group [as described in (Dennis *et al.*, 2003; Huang da *et al.*, 2009a, b)]. All other analyses were made using Microsoft Excel software.

6.18 Solutions

Phosphate buffered saline (PBS): 171mM NaCl, 3.3M KCl, 10.1mM Na₂PO₄, 1.8 mM KH₂PO₄

Culturing medium: RPMI medium with L-glutamine (PAA laboratories GmbH), Fetal bovine serum (Sigma) (10% v/v), Penicillin (1% v/v), Glutamax-I 100x (GIBCO) (1% v/v)

Immunoprecipitation (IP) buffer: 150mM NaCl, 50mM Tris-HCl (pH 7.5), 5mM EDTA, NP-40 (0.5% v/v), Triton-X-100 (1.0% v/v). When necessary, add freshly 0.5µl P8430 Protease inhibitor cocktail (Sigma) and 5µl 0.1M PMSF in isopropanol per 1 ml of IP buffer

Gey's stock A: 35.0g KCl, 0.56g NaHPO₄ and 5.0g glucose in one litre dH₂O.

Gey's stock B: 4.2 g MgCl₂.6H₂O and 1.4g MgSO₄.7H₂O in one litre dH₂O.

Gey's stock C: 22.5g NaHCO₃ in one litre dH₂O.

APPENDIX

Supplementary figures

Figure S.1

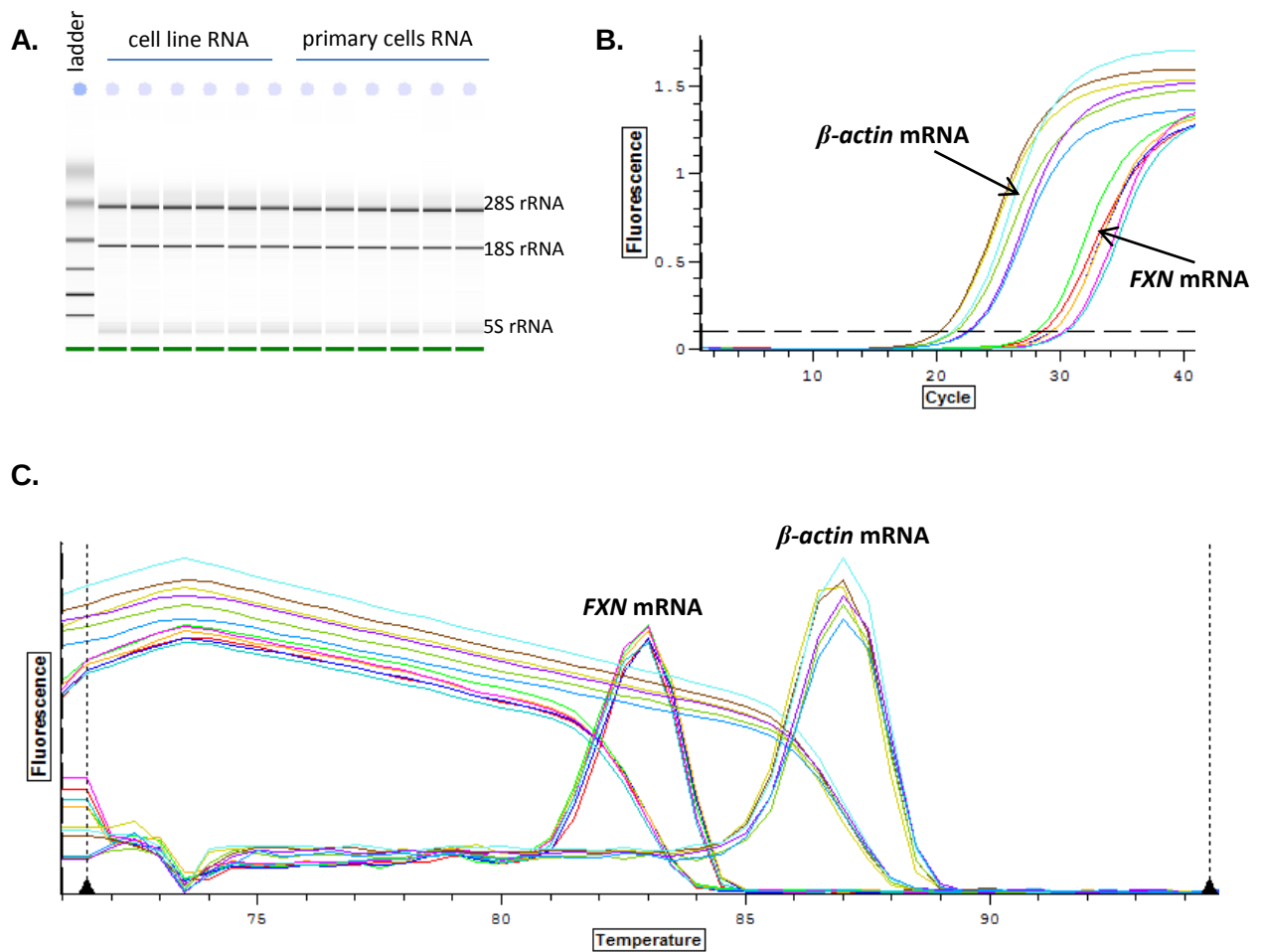


Figure S.1 – Representative RNA gel and Q-RT-PCR signals for *FXN* expression. [A] RNA gel. Total RNA was extracted with Trizol® and its quality was checked by Agilent Bioanalyser RNA-nano microgel throughout. Intact 28S, 18S and 5S rRNA bands could be clearly seen. The average RNA integrity was calculated ~95% by the Agilent software for the above-shown samples. [B] Q-RT-PCR quantitation curve. Curves for the PCR products corresponding to *FXN* and β -actin mRNA are indicated. [C] Melting curves for *FXN* and β -actin mRNA primers.

Figure S.2

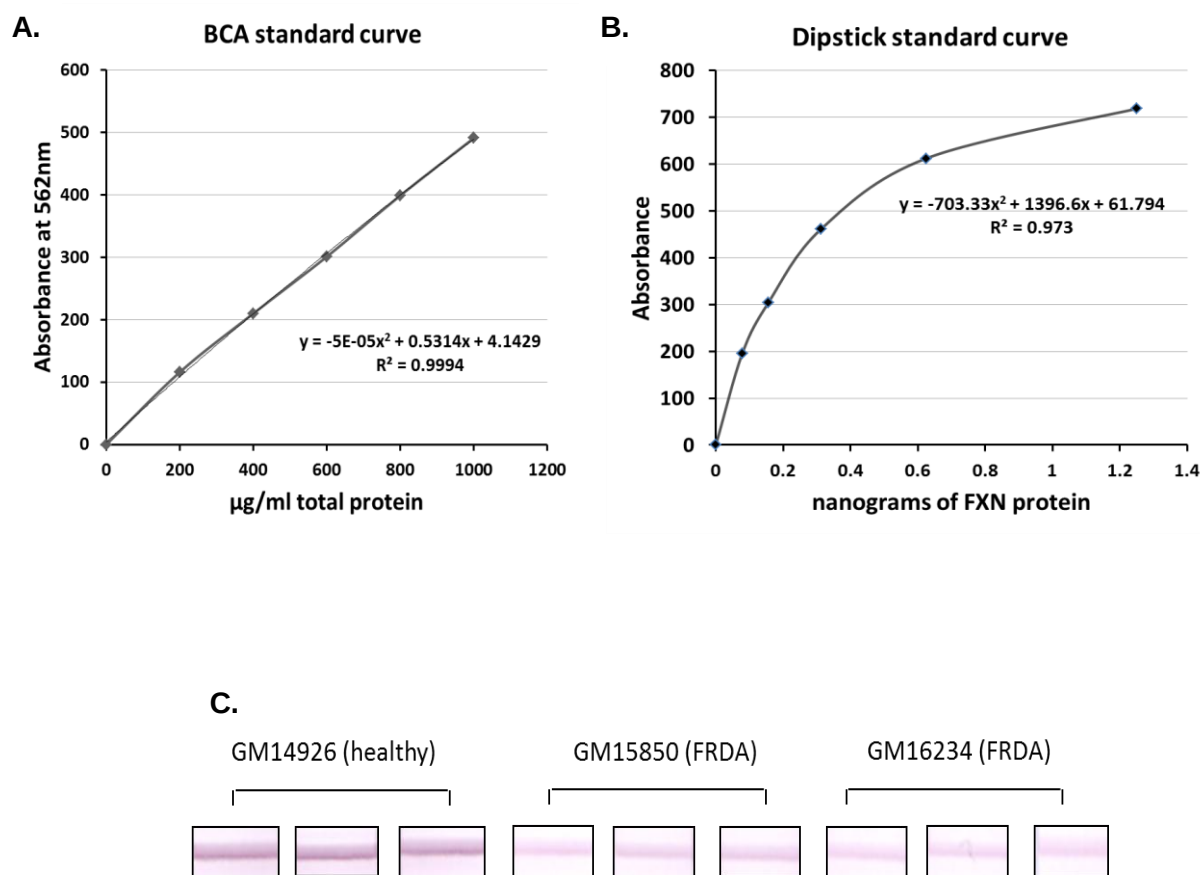


Figure S.2 – Mitosciences dipstick assay for FXN protein. [A] A representative BCA protein quantitation standard curve. This curve was used to make sure that the same levels of total protein were assayed from each sample. [B] A representative standard curve for FXN dipstick absorbance. The standard curve was drawn using the signal obtained from recombinant FXN protein, as detected via the MS1000 dipstick reader. This curve was used to calculate the FXN protein levels by interpolation. [C] Dipstick pictures. Differences in the FXN-based colourimetric signal could sometimes be clearly distinguished by eye. As can be seen, FRDA lymphoblastoid cell lines express much less FXN protein compared to the healthy cell line. These pictures correspond to the result presented in figure 2.2B.

Figure S.3

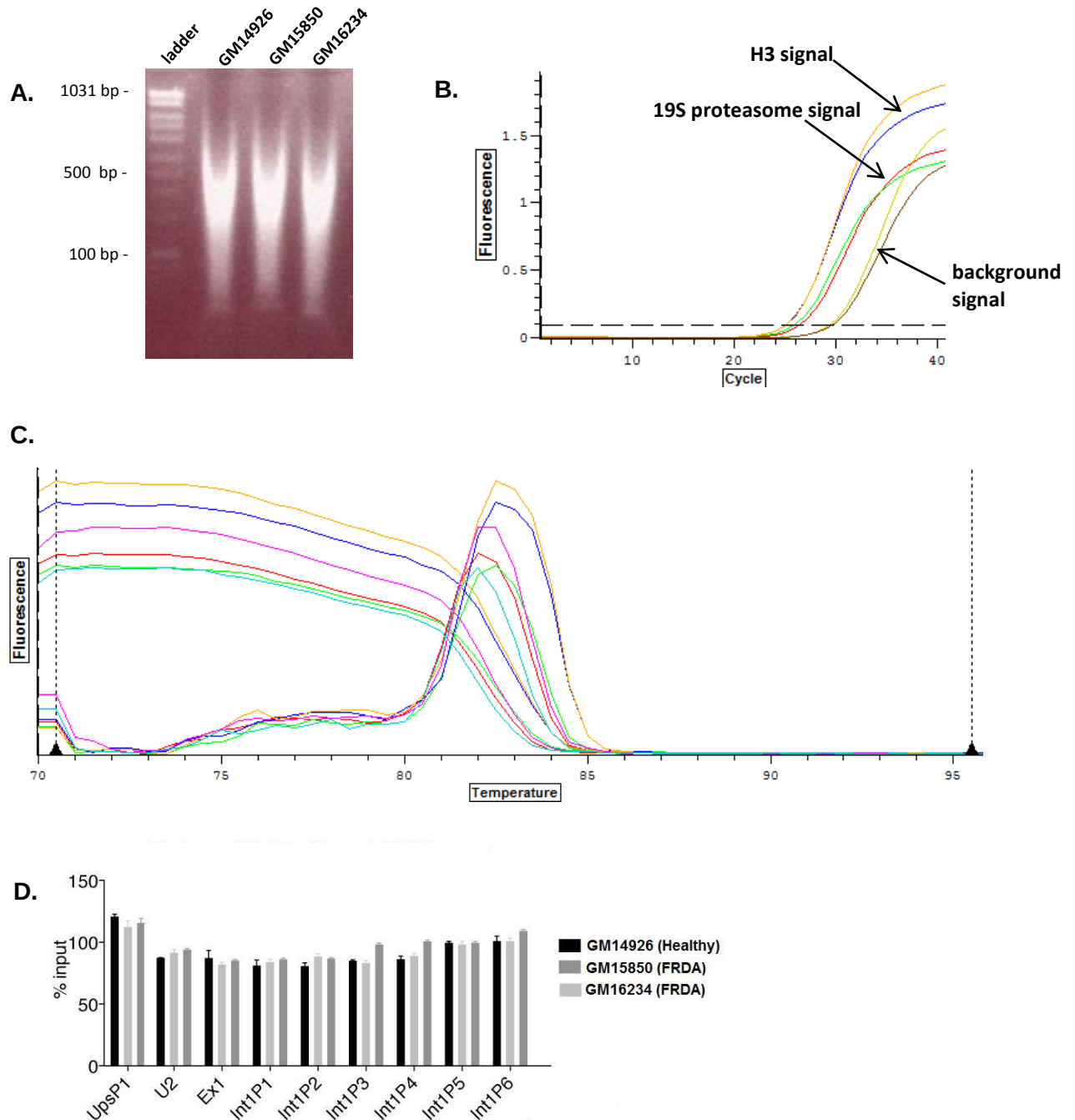


Figure S.3 – Representative sonication gel, ChIP Q-RT-PCR signals and H3 levels at the *FXN* locus. [A] **Sonication gel.** Whole chromatin obtained from the fixed nuclei was sonicated in Diagenode Bioruptor® at ‘high energy’ for 1 hours and 30 minutes (24s on and 30s off) to obtain a fragmented chromatin between 200 and 500 bp. [B] **Q-RT-PCR quantitation curve of the Int1P4 primer for ChIP against the proteasome.** As indicated, background signal clearly had a higher C(t) compared to the antibody-immunoprecipitated material. [C] **Melting curve for the Int1P4 primer.** [D] **H3 levels at the first part of the *FXN* locus.** ChIP with pan-H3 antibody revealed that H3 levels are almost flat throughout the first part of the *FXN* locus. [Signal was shown as percentage to input DNA. Error bars: SEM , n=3]

Figure S.4

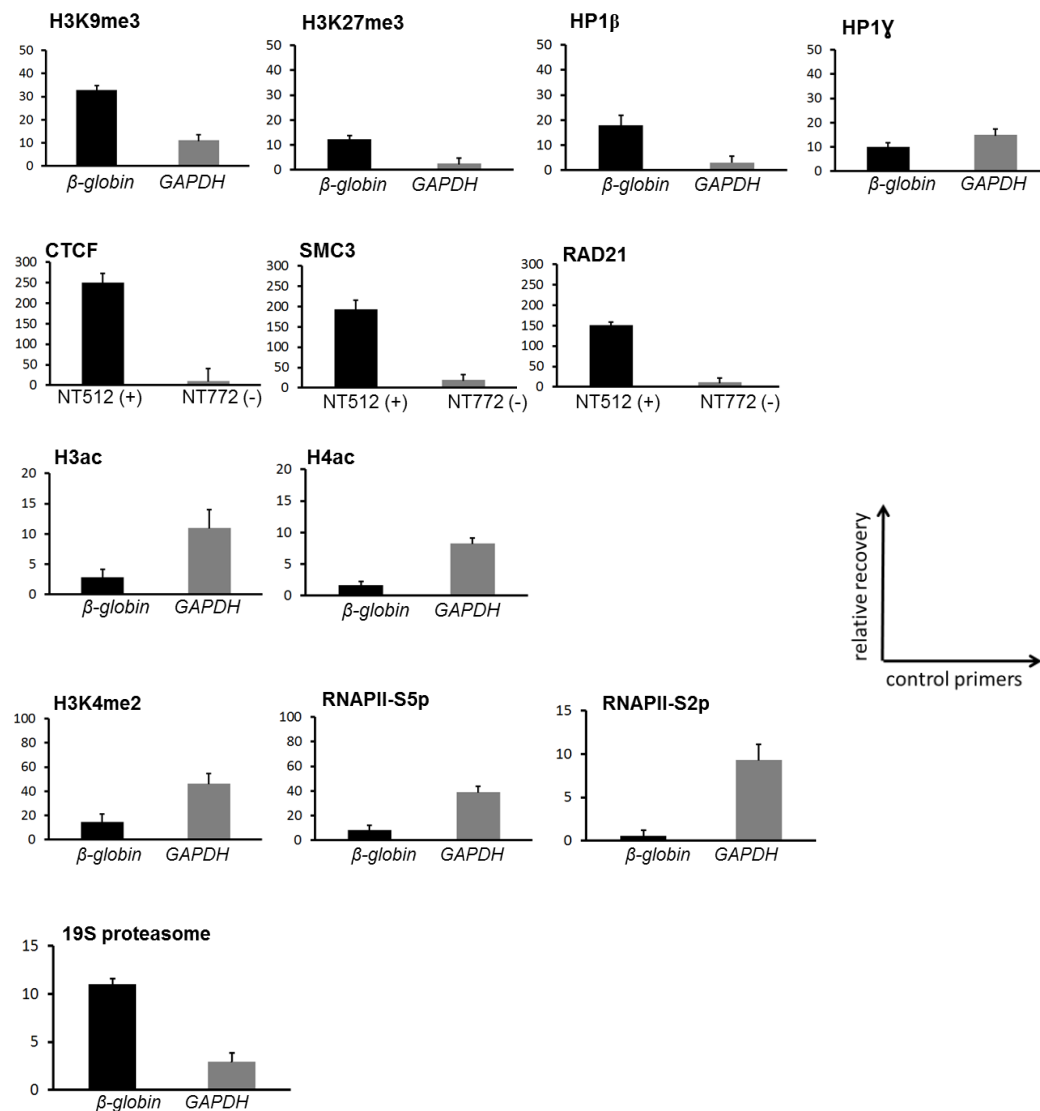


Figure S.4 – Controls for chromatin immunoprecipitation. In order to test the specificity of ChIP and check the loading differences, immunoprecipitated material with the above-indicated antibodies were tested throughout the experiments for a positive and negative ChIP signal. For most of them, β -globin was used as a control to represent an inactive gene whereas the housekeeping gene GAPDH was used to represent an active gene. For CTCF and cohesin subunits, two genomic fragments were amplified as a control signal. These fragments were previously shown to have a high (+) and low (-) enrichment for CTCF and cohesin binding in a published study (Wendt *et al.*, 2008). [ChIP signal was shown as recovery percentage relative to H3. Error bars: SEM, n>5.]

Figure S.5

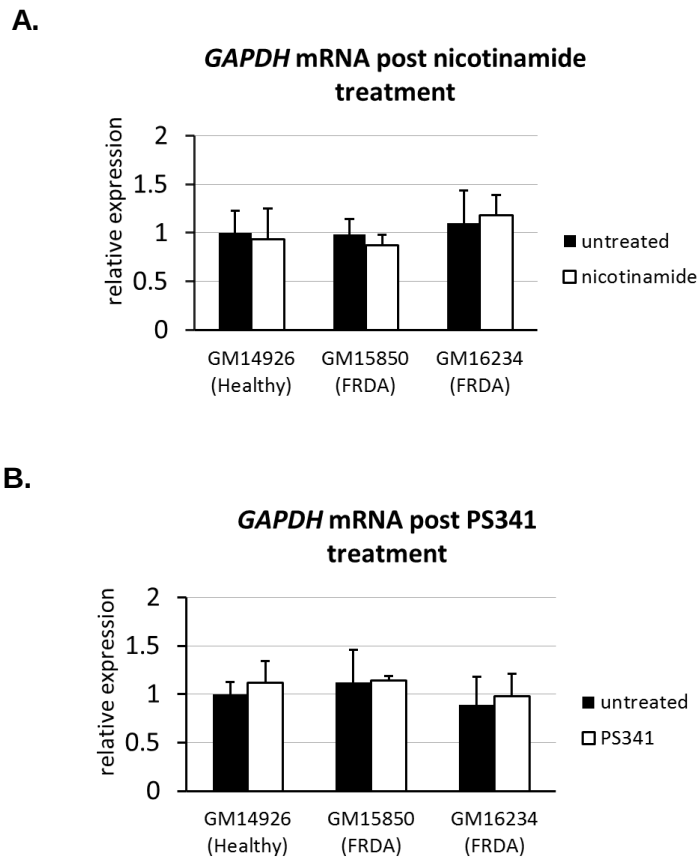


Figure S.5 – Response of *GAPDH* expression to nicotinamide or PS341 treatments. In order to test the specificity of the effect observed by nicotinamide and PS341 treatments on the *FXN* expression, *GAPDH* mRNA levels were evaluated on the lymphoblastoid cell lines via Q-RT-PCR. **[A] Nicotinamide treatment.** 10mM nicotinamide treatment for 3 days did not effect *GAPDH* levels significantly. **[B] PS341 treatment.** 10 μ M PS341 treatment for 8 hours did not interfere with the *GAPDH* expression levels as shown. [Results were presented as relative expression to untreated healthy cell line. Normalisation was done against β -actin expression. Error bars: SEM, n=3.]

Figure S.6

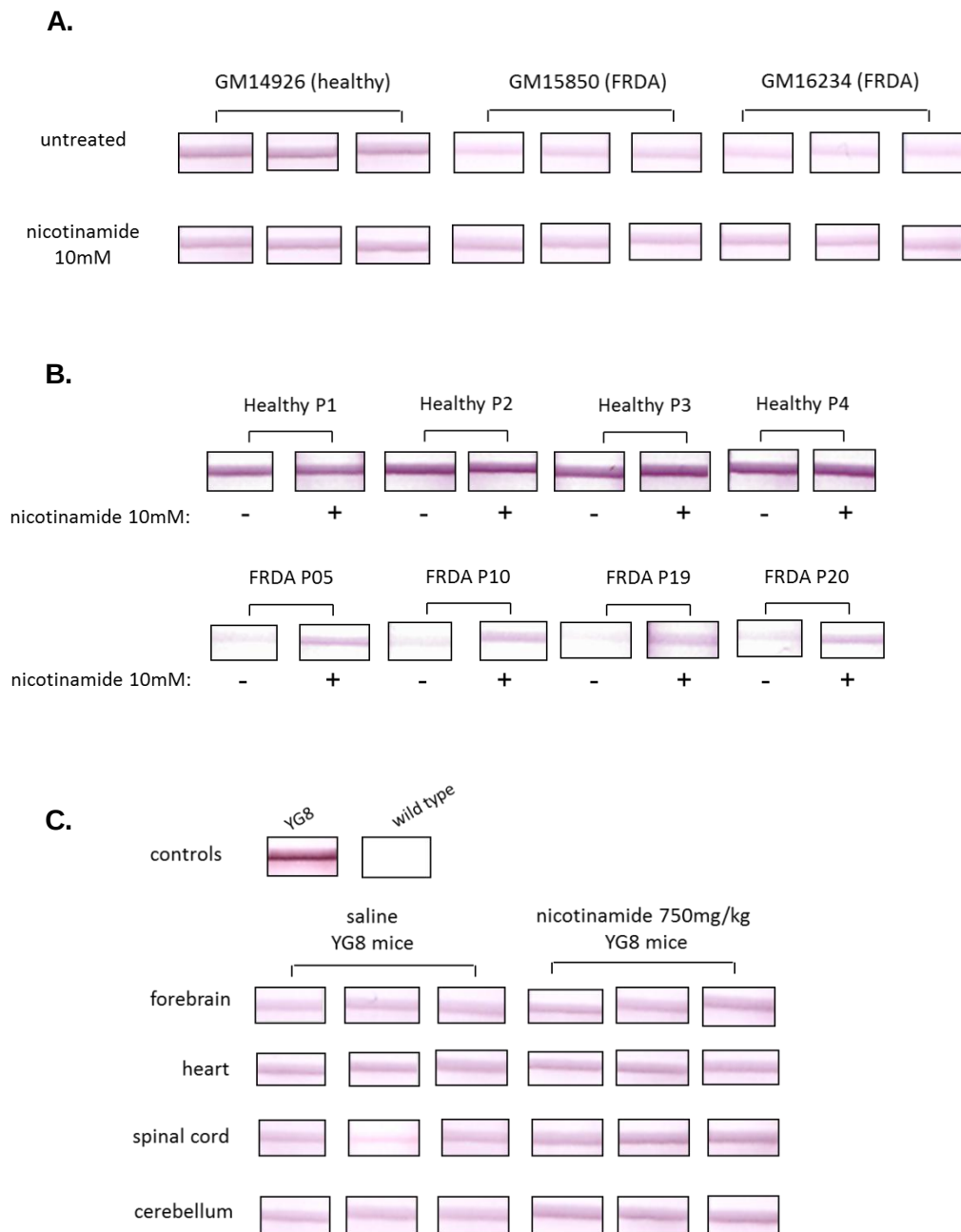


Figure S.6 – Dipstick pictures for FXN protein levels post-nicotinamide treatment. Cells were treated with nicotinamide (10mM) for three consecutive days. **[A] FXN levels in lymphoblastoid cell lines.** Nicotinamide upregulated FXN protein levels significantly in FRDA cell lines. However, a downregulation effect was observed for the healthy cell line. **[B] FXN levels in primary lymphocytes.** Nicotinamide upregulated FXN protein levels significantly in FRDA cells whereas no obvious effect was observed on the healthy cells. **[C] FXN levels in YG8 mouse tissues.** Nicotinamide injection (750 mg/kg for 5 consecutive days) upregulated FXN levels significantly in nerve tissues but not in heart. [5µg of total protein was used for cell lines and primary cells as well as nerve tissues from YG8 mice. 3µg of total protein was used for heart.]

Figure S.7

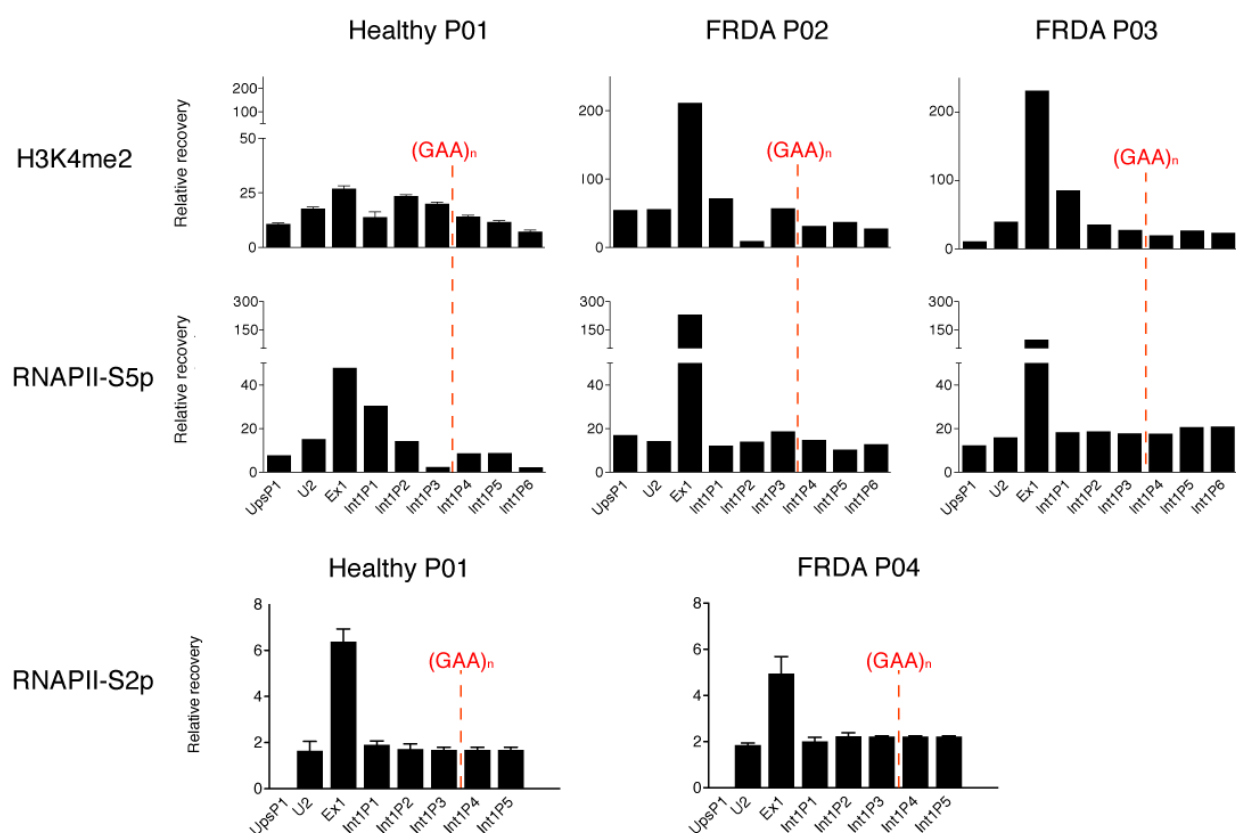


Figure S.7 – H3K4me2 and RNAPII levels at the first part of *FXN* locus in primary lymphocytes.

ChIP was performed on the healthy and FRDA primary lymphocytes using highly specific antibodies against the newly transcribed chromatin mark H3K4me2 and initiating (S5p) and elongating (S2p) forms of RNAPII. Both H3K4me2 and RNAPII levels peaked in Ex1 of all cells at similar levels. [ChIP signal was shown as recovery percentage relative to H3. Error bars: SEM, n=3.]

Figure S.8

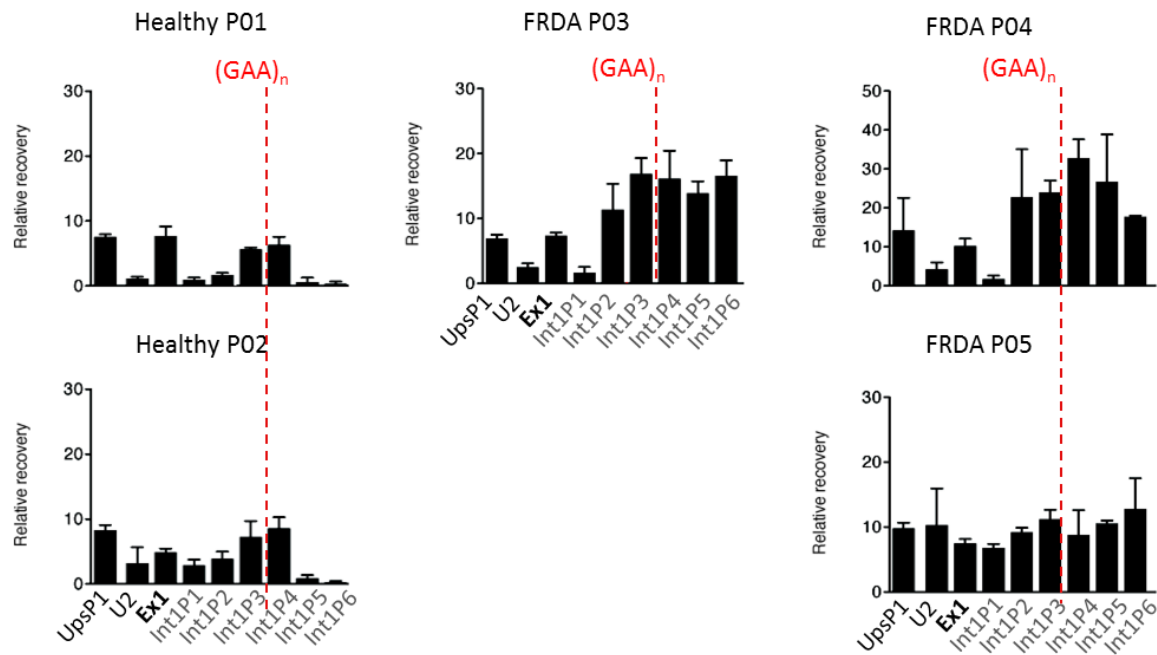


Figure S.8 - Proteasome ChIP with primary lymphocytes. The first part of the *FXN* locus was investigated for proteasome binding using ChIP against the 19S proteasomal subunit RPN10. Binding was significantly enriched in primary cells obtained from FRDA patients compared to healthy. Interestingly, the distribution of proteasome signal resembles that of H3K9me3 and the HP1 binding presented in figure 2.3. [ChIP signal was shown as recovery percentage relative to H3. Error bars: SEM, n=3]

Figure S.9

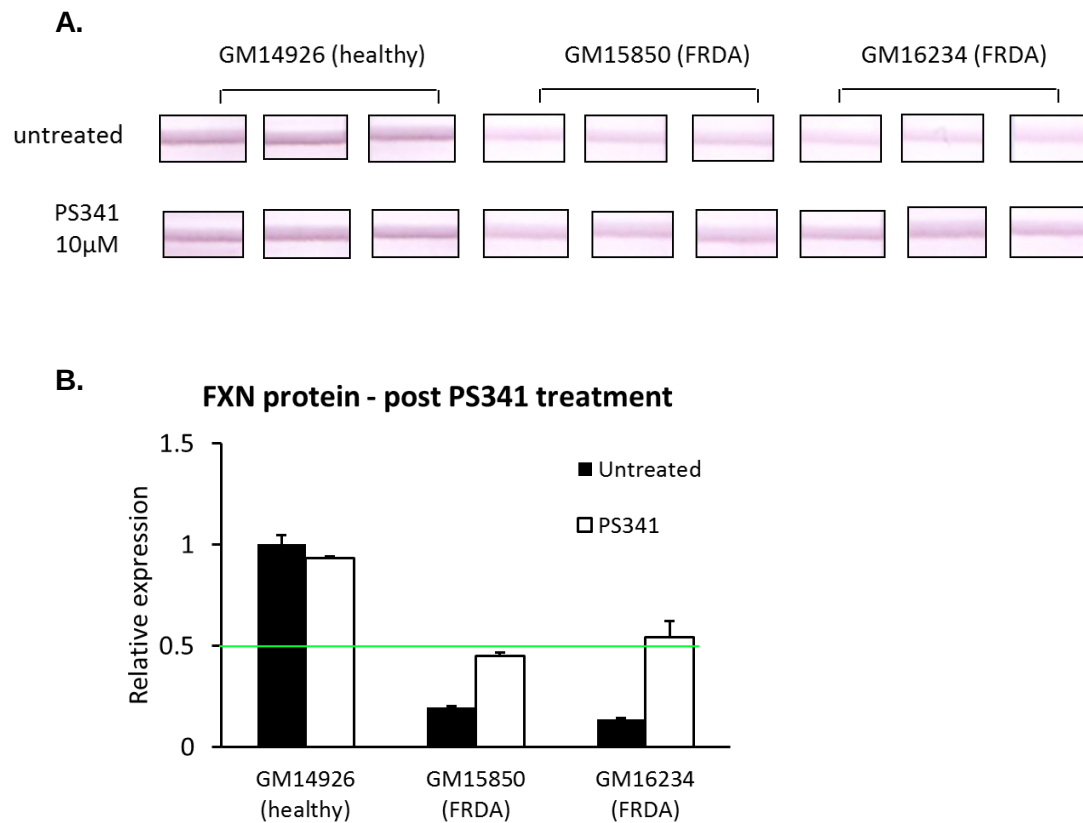


Figure S.9 – FXN protein levels post-PS341 treatment. Here treatment was with the proteasome inhibitor PS341 at a concentration of 10µM for 24 hours in order to measure the effect on *FXN* expression at the protein level. **[A] Dipstick pictures.** **[B] Dipstick analysis.** PS341 treatment upregulated *FXN* protein levels in FRDA cell lines up to potentially therapeutic levels; in agreement with the RNA expression result presented in figure 2.12. [Data was presented as relative expression to the value obtained from untreated healthy cells. Green line indicates the relative expression levels of asymptomatic heterozygous FRDA carriers. Error bars: SEM, n=3.]

Figure S.10

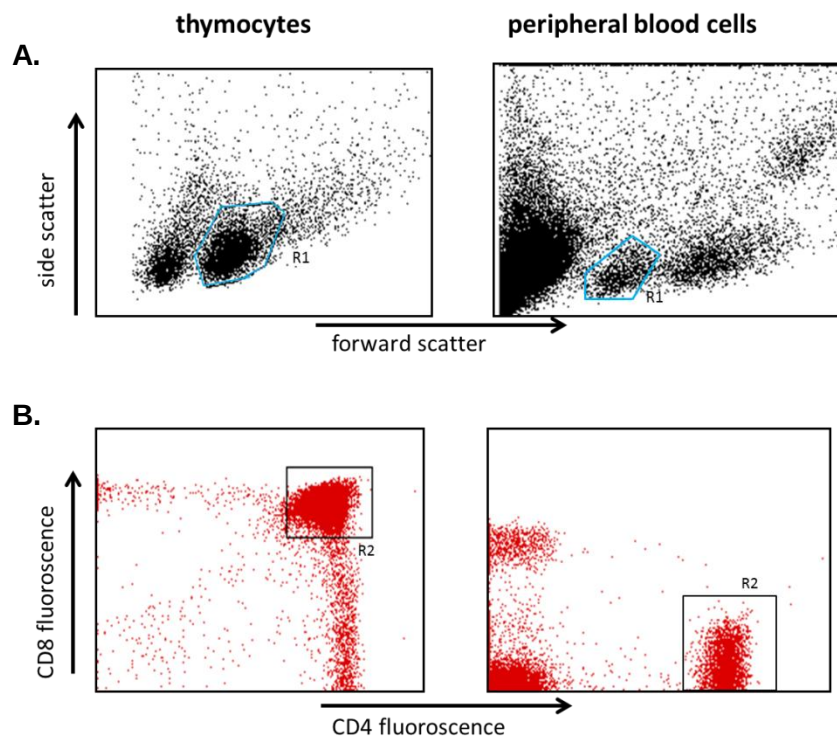


Figure S.10 – Representative FACS dot-plots for selecting a uniform cell population.
[A] Acquisition dot-plots. Intact thymocytes and peripheral lymphocytes were selected.
[B] CD4/CD8 fluorescence dot-plots. For thymocytes, 20,000 CD4 and CD8 double-positive cells were gated and examined for hCD2 fluorescence. For peripheral blood cells, 20,000 CD4 single-positive cells were gated. [Left panels for thymocytes and right panels for peripheral blood cells. Each dot represent a single cell.]

Figure S.11

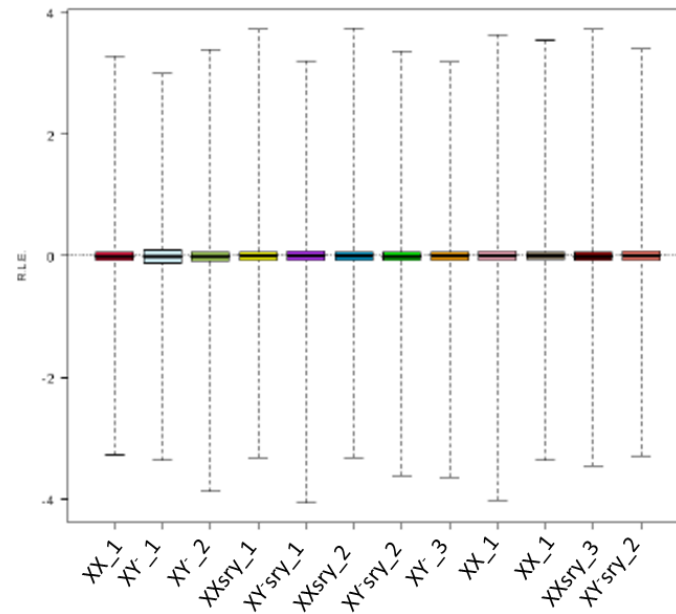


Figure S.11 – Relative log₂ expression (RLE) plot for microarrays. RLE is the log₂ scale estimate of expression on each array relative to the median value across arrays on a probe set by probe set basis. Each box represents one array. The boxes are all around 0 and the variation levels are around the same levels for each array. This confirms that the arrays are experimentally comparable. [RLE plot was drawn with R Bioconductor after robust multichip average (RMA) normalisation.]

Figure S.12

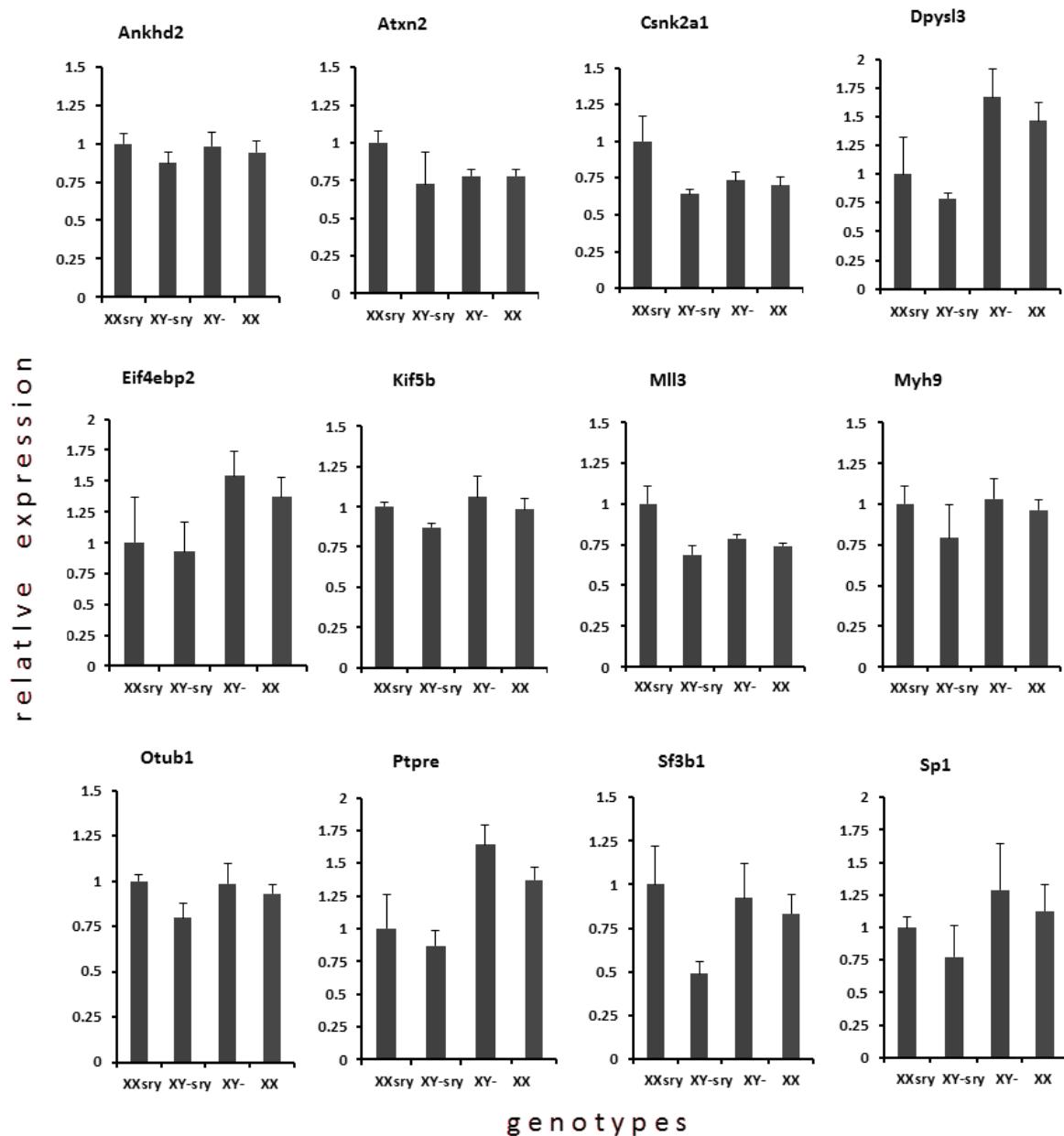


Figure S.12 – Q-RT-PCR validation of sex chromosome complement-sensitive genes. mRNA levels of 16 sex chromosome-sensitive genes which show the highest fold change on microarrays (XXsry> XY-sry and XY->XY-sry) were analysed and 12 of them were validated. [The same total RNA samples from the microarray experiments were used. Expression was presented relative to XXsry and housekeeping normalisation was done against *Hmbs* and β -actin mRNA. Error bars: SEM, three individual mouse-samples per group.]

Figure S.13

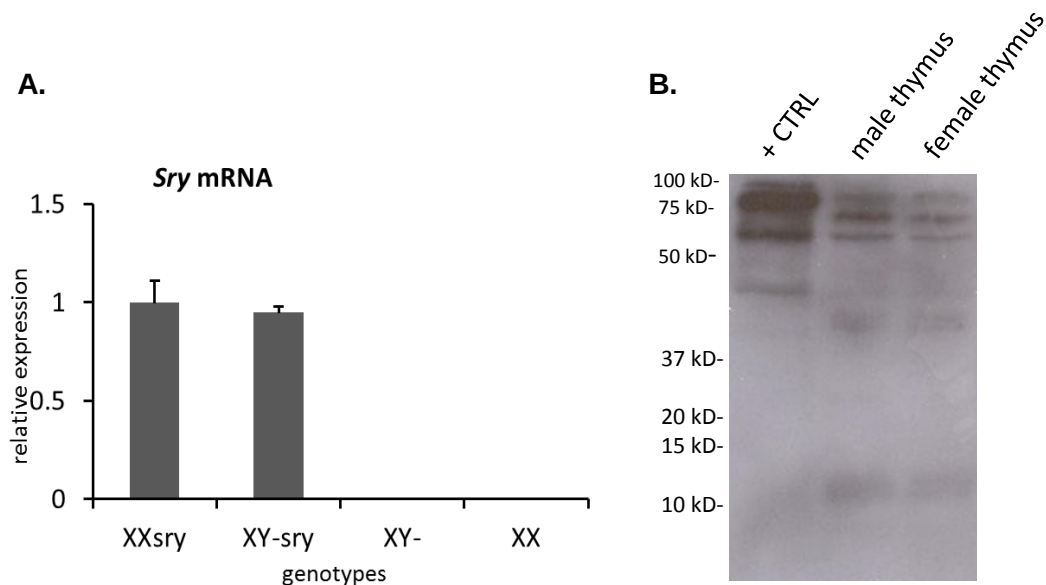


Figure S.13 – Analysis of Sry expression in mouse thymus. [A] Q-RT-PCR analysis. Sry mRNA was detected in thymus tissue of 6-8 week old XXsry and XY^{sry} male mice whereas no signal was obtained from XY⁻ and XX female mice. [Expression was presented relative to XXsry and housekeeping normalisation was done against *Hmbs* and β -actin mRNA. Error bars: SEM, three individual mouse-samples per group.] [B] Sry western blot. The banding pattern in this western blot is consistent with the study of Bradford *et al.* (2007), where they stated that Sry band should appear at 55 kD with no female control. Unfortunately, no male specific Sry band was detected here in mouse thymus tissue using the α -Sry antibody (Bradford *et al.*, 2009; Bradford *et al.*, 2007; Wilhelm *et al.*, 2005). Therefore, the antibody may cross-react with another protein on western blots, possibly with one of the Sox proteins that have similar amino acid compositions and structures. [Positive control, which is the total protein extract of Sry over-expressing HEK293T cells, was kindly provided by Patrick Wijchers. The blot picture is representative for three independent experiments.]

Supplementary tables

Table S.1

List of sex chromosome complement-sensitive (SCS) genes

100039204A5Rik	Ash1l	D19Ertd737e	Hook1	Pdzd8	Sfrs11	Zdhhc21
100040086G2Rik	Atr	D3Ertd300e	Hspa9	Phb2	Sfrs12	Zfp101
1110003F05Rik	Atxn1	D5Ertd579e	Id2	Phf2011	Sh3rf1	Zfp148
1200003C05Rik	Atxn2	D6Wsu176e	Ifna5	Phf7	Skp2	Zfp187
1500005K14Rik	AU015263	D7Ertd602e	Ifnar1	Phip	Slc25a30	Zfp644
1700012H17Rik	B3galnt2	D930017J03Rik	Igk-V1	Phxr4	Slc4a7	Zfr
1700081L11Rik	B3gnt1	D9Ertd306e	Il10ra	Pigt	Sltm	Zkscan3
1810014B01Rik	B430201A12Rik	Ddah1	Il1rap	Pik3cg	Smarca4	Zmiz1
2310001H17Rik	B930025B16Rik	Ddi2	Inpp4b	Pik3r1	Snrpd3	Znrf1
2310047O13Rik	Bach1	Ddx6	Inpp5f	Pip5k1a	Snw1	
2410042D21Rik	Bat2d	Dmtf1	Itch	Pisd	Snx27	
2810474O19Rik	BC016423	Dnaja2	Jmjd3	Pknex1	Snx29	
2900010J23Rik	BC028789	Dnajc1	Jrk	Plod2	Solh	
3110045A19Rik	BC031353	Dock8	Khsrp	Ppap2b	Sox4	
3110057O12Rik	Bicc1	Dopey2	Kif5b	Ppp1cb	Sp1	
4632427E13Rik	Bicd2	Dpysl3	Klf3	Ppp2r5a	Spa17	
4633401B06Rik	Birc6	Dtl	Klf7	Prcc	Spnb2	
4832420A03Rik	Bmp2k	Dync1h1	Klhl24	Prpf19	St6gal1	
4921513D23Rik	Bptf	E130308A19Rik	Krit1	Prpf38a	Stambpl1	
4930402E16Rik	Braf	E230029C05Rik	Larp4	Prpf38b	Stat3	
4932438A13Rik	Brd2	Edem3	Lmnbl	Psap	Stx6	
4933434E20Rik	Brd4	EG633640	Lrba	Psip1	Sympk	
5830407E08Rik	Btg2	Ehd2	Lss	Psmc13	Taf15	
5830417C01Rik	C030034I22Rik	Eif4ebp2	Luc7l2	Pten	Taok1	
5830436I19Rik	C030043A13Rik	Elac1	Ly6e	Ptprd	Tatdn1	
5830457O10Rik	C130070B15Rik	Elavl1	Ly6k	Ptpre	Tbrg4	
6030458C11Rik	C130098B18Rik	Elf1	Malat1	Pum1	Tcra	
6330417A16Rik	C330006A16Rik	Elf2	Map4k4	Purb	Timp2	
6430510B20Rik	C330006D17Rik	Eml5	Matr3	Pus3	Tle3	
6430510M02Rik	C430003N24Rik	Enpp5	Mbnl2	Rab5b	Tlk1	
6720418B01Rik	C430014K11Rik	ENSMUSG00000058045	Mbp	Rab5c	Tln1	
6720467C03Rik	C76213	ENSMUSG00000058380	Mcoln1	Rad54l	Tmcc1	
6820431F20Rik	C78516	ENSMUSG00000073237	Me2	Rap2b	Tmem57	
8030494B02Rik	C80258	ENSMUSG00000073981	Mex3c	Rapgef3	Tnrc6b	
9030612M13Rik	C920008N22Rik	Esf1	Mga	Rbbp4	Tns1	
9130009I01Rik	Cald1	Fbxl11	Mical3	Rbm39	Tor1aip1	
9130404D08Rik	Camk1d	Fcho2	MI3	Rbm4	Tpr	
9530029O12Rik	Ccdc58	Flnb	MI5	Rbm5	Tra2a	
A130010C12Rik	Ccdc76	Fryl	Mon1b	Rc3h2	Traip	
A130012E19Rik	Ccdc88a	Fscn1	Msi2	Rcor1	Trim41	
A430061O12Rik	Ccdc88c	Fusip1	Msl2l1	Rest	Trps1	
A630005I04Rik	Ccny	Fut8	Mtss1	Rhoh	Tsc22d1	
A630023P12Rik	Ccr6	G3bp2	Myh9	Rinl	Twistnb	
A630033E08Rik	Cd4	Gas2l3	Mysm1	Rnd3	Ubn1	
A930001N09Rik	Cd93	Gdap10	Napg	Rnf125	Usp7	
A930041I02Rik	Cdc37l1	Gfra2	Nbeal1	Rnf166	Vldlr	
Ahctf1	Cdca7	Ggnbp2	Ncoa2	Rnf169	Wdr35	
Ahsa1	Cdk7	Grin2b	Ndel1	Rnmt	Wdr68	
AI314760	Cep97	Gsk3b	Nek4	Rps6kb1	Whsc1l1	
AI426953	Clasp1	Gtf3c2	Nfat5	Rraga	Xpnpep3	
Alkbh8	Cnot6l	Gtpbp2	Nipbl	Rreb1	Xpo4	
Ambra1	Copa	Guf1	Nisch	Rsf1	Xrn1	
Angel2	Cpsf6	H13	Nr2c1	Runx2	Yars2	
Ankhd1	Cr1l	Hectd1	Nrip1	Saps3	Yipf4	
Anxa9	Csnk1a1	Herc4	Otub1	Sbno1	Ywhab	
Ap4e1	Csnk2a1	Herc5	Pcgf5	Sec62	Ywhaz	
Aph1a	Csnk2a2	Hhip	Pcgf6	Senp1	Zbtb20	
Arglu1	Ctnnd2	Hibadh	Pdcd6ip	Setd6	Zc3h4	
Arhgap30	D13Ertd787e	Hist1h2ae	Pdia3	Sf3b1	Zcchc7	
Arpc2	D19Ertd409e	Hnrnpab	Pdpk1	Sfi1	Zdhhc20	

* The bold gene names indicate those which are expressed differently between normal males and females. These are 175 sexually dimorphic SCS genes introduced in Figure 4.5C.

Table S.2

List of antibodies

antibody /clone name	species used	description/isotype	purpose / comment	company	cat #
anti-H3	Hs	rabbit polyclonal/ IgG	ChIP (5µg/per sample)	Abcam	Ab1791
anti-H3K9me3	Hs and Mm	rabbit polyclonal/ IgG	ChIP (6µg/per sample) and IF (1:100)	Millipore	17-625
anti-H3K27me3	Hs	mouse monoclonal/ IgG3	ChIP (6µg/per sample)	Abcam	Ab6002
anti-H3K4me2 (Y47)	Hs	rabbit monoclonal/ IgG	ChIP (6µg/per sample)	Abcam	Ab32356
anti-H3ac / K9+K14+K18+K23+K27	Hs	rabbit polyclonal/ IgG	ChIP (6µg/per sample)	Abcam	Ab47915
anti-H4ac / K5+K8+K12+K16	Hs	rabbit polyclonal/ IgG	ChIP (6µg/per sample)	Millipore	06-866
anti-HP1β (1MOD-1A9)	Hs	mouse monoclonal/ IgG1	ChIP (8µg/per sample)	Millipore	MAB3448
anti-HP1γ (42S2)	Hs and Mm	mouse monoclonal/ IgG1	ChIP (8µg/per sample)	Millipore	05-690
anti-CTCF	Hs	mouse monoclonal/ IgG1k	ChIP (6µg/per sample)	Millipore	17-1044
anti-SMC3	Hs	rabbit polyclonal/ IgG	ChIP (6µg/per sample)	Abcam	Ab9263
anti-RAD21	Hs	rabbit polyclonal/ IgG	ChIP (6µg/per sample)	Abcam	Ab992
anti-RNAPII-S5p (4H8)	Hs and Mm	mouse monoclonal/ IgG1	ChIP (6µg/per sample)	Abcam	Ab5408
anti-RNAPII-S2p (H5)	Hs	mouse monoclonal/ IgGM	ChIP (10µg/per sample)	Abcam	Ab24758
anti-Proteasome 19S RPN10 (AH1.1)	Hs	mouse monoclonal/ IgG1	ChIP (8µg/per sample)	Abcam	Ab20239
anti-Proteasome 20S C2	Hs	rabbit polyclonal/ IgG	ChIP (8µg/per sample) -low signal	Abcam	Ab3325
anti-Proteasome 20S (LMP7)	Hs	rabbit polyclonal/ IgG	ChIP (8µg/per sample) -no signal	Abcam	Ab3329
anti-Proteasome 20S (MCP231)	Hs	mouse monoclonal/ IgG1	ChIP (8µg/per sample) -no signal	Abcam	Ab22674
anti-Proteasome 19S Rpt6 (p45-110)	Mm	mouse monoclonal/ IgG2b	ChIP (8µg/per sample)	Enzo Life S.	BML-PW9265
anti-Proteasome 20S β6	Mm	rabbit polyclonal/ IgG	ChIP (8µg/per sample) and IF (1:100)	Enzo Life S.	BML-PW9000
anti-MSI2 (EP1305Y)	Mm	rabbit monoclonal/ IgG	FACS (1:100)	Abcam	Ab76148
anti-CSNK2A1 (EP1963Y)	Mm	rabbit monoclonal/ IgG	FACS (1:100)	Abcam	Ab76040
anti-CD93-PE con.	Mm	rat monoclonal/ IgG2b κ	FACS (1:100)	Biolegend	136503
anti-Kremen1	Mm	goat polyclonal /IgG	FACS (1:100)	R&D Systems	AF1647
anti-SRY (α)	Mm	rabbit polyclonal/ IgG	Western blot (1:500)	by Peter Koopman *	
anti-hCD2 (for human)-FITC con.	Mm (t.genic)	mouse monoclonal /IgG2a	FACS (1:100)	Invitrogen	CD0201-4
anti-CD4 (GK1.5) - PE con.	Mm	rat monoclonal/ IgG2b	FACS (1:100)	Abcam	Ab86859
anti-CD8 (53-6.7) - APC con.	Mm	rat monoclonal /IgG2a	FACS (1:100)	Abcam	Ab25499
Secondary to rabbit IgG-FITC con.	Mm	goat polyclonal /IgG	FACS (1:100)	Abcam	Ab97050
Secondary to goat IgG-FITC con.	Mm	donkey polyclonal / IgG	FACS (1:100)	Abcam	Ab97109
Secondary to rabbit IgG-HRP con.	Mm	donkey polyclonal / IgG	Western blot (1:3000)	Abcam	Ab97064

Hs: Homo sapiens
Mm: Mus Musculus

* Personal communication, The University of Queensland, Australia

[The species used here are not necessarily all the species that the antibody had been tested by the company. It only refers to the species used in this thesis.]

Table S.3

List of primers for chapter 2

species	primer name	purpose	product (bp)	primer sequence (5' to 3')
Hs	β actin_E3E4_L	mRNA	327	GCGGGAAATCGTGCGTGACAT
	β actin_E3E4_R			GATGGAGTTGAAGGTAGTTTCGTG
Mm	β actin_Ex2_L	mRNA	205	CTTTTCACGGTTGGCCTTAG
	β actin_Ex2_R			GGACTCCTATGTGGGTGACG
Hs	GAPDH_Ex1Ex5_L	mRNA	307	GGTCGTATTGGGCGCCTGGT
	GAPDH_Ex1Ex5_R			CCTGCAAATGAGCCCCAGCCTT
Hs	FXN_Ex1Ex5_L	mRNA	376	GACATCGATGCGACCTG
	FXN_Ex1Ex5_R			CAGTCCAGTCATAACGC
Hs	FXN_Ex3Ex4_L	mRNA/YG8 mice	140	ATGTCTCCTTGGGAGTGGTGTCT
	FXN_Ex3Ex4_R			CCCAGTCCAGTCATAACGCTTAGGT
Hs	FXN_Int3Ex4_L	primary RNA	101	TTCCACCTAATCCCCTAGAGTG
	FXN_Int3Ex4_R			AGCCAGATTGCTTGTTTGG
Hs	FXN_UpsP1_L	ChIP	190	GGGATTTCTTTTCCCAGAG
	FXN_UpsP1_R			ACCTTGCAGGACACCAAAAC
Hs	FXN_U2_L	ChIP	127	CCCCACATACCCAATGCTG
	FXN_U2_R			GCCCGCCGCTTCTAAATTC
Hs	FXN_Ex1_L	ChIP	189	GGAGCAGCATGTGGACTCTC
	FXN_Ex1_R			CGGCGCGGATACTTACTG
Hs	FXN_Int1P1_L	ChIP + RIP	155	CTCCCGGTTGCATTTACACT
	FXN_Int1P1_R			GTGACAAGCATGGAGACAGC
Hs	FXN_Int1P2_L	ChIP	123	CTGACCCGACCTTTCTTCCA
	FXN_Int1P2_R			TGGGCGTCACCTTTATCTTC
Hs	FXN_Int1P3_L		116	GAAACCCAAAGAATGGCTGTG
	FXN_Int1P3_R	ChIP		TTCCCTCCTCGTGAAACACC
Hs	FXN_Int1P4_L		117	CTGGAAAAATAGGCAAGTGTGG
	FXN_Int1P4_R			CAGGGGTGGAAGCCCAATAC
Hs	FXN_Int1P5_L	ChIP	177	CCCTTGACATCTTGGGTAT
	FXN_Int1P5_R			GAGAAAAGGGTGGGGAAGAG
Hs	FXN_Int1P6_L		172	AGCCCCACATTCTCAGACAC
	FXN_Int1P6_R	ChIP		ACGCACCAAAGGGTAACCTG
Hs	β globin_Ex2_L		150	GCTGGTGGTCTACCTTGGA
	β globin_Ex2_R			AGGTTGTCCAGGTGAGCCAG
Hs	GAPDH_Ex4_L	ChIP	134	CACCGTCAAGGCTGAGAACG
	GAPDH_Ex4_R			ATACCCAAGGGAGCCACACC
Hs	RIP1_L	RIP	157	GGAGCAGCATGTGGACTCTC
	RIP1_R			CAGGTCGCATCGATGTCTG

Hs: Homo sapiens
Mm: Mus Musculus

Table S.4

List of primers for chapter 3

species	primer name	purpose	product (bp)	primer sequence (5' to 3')
Hs	ALDOA_Ex1_L	ChIP+RNA	211	GCTGCTACCACACACAAGT
	ALDOA_Ex1_R			AATCTCCCTCCTCCCTTGAC
Hs	Alu_L	ChIP+RNA	124	GCCTGTAATCCCAGCACTTT
	Alu_R			CGCCCGGCTAATTTTGTAT
Hs	γ-sat_L	ChIP+RNA	95	CAGGGTGCCTGTGTCTCTC
	γ-sat_R			CAGGGTGCCTGTGTCTCTC
Hs	HMBS_Ex1_L	ChIP+RNA	190	CTCTGCGGAGACCAGGAGT
	HMBS_Ex1_R			CGCTTGGAAAGTAGGCTGTG
Hs	HP_Ex1_L	ChIP+RNA	214	CAGAAAAATGCAACAGCGAAA
	HP_Ex1_R			TAACCCACACGCCCTACTTC
Hs	HPRT_Ex1_L	ChIP+RNA	102	CCCTCAGGCGAACCTCTC
	HPRT_Ex1_R			CTGACTGCTCAGGAGGAGGA
Hs	L1_5UTR_L	ChIP+RNA	137	TCACTCCCGCCCTAATACTG
	L1_5UTR_R			TTTGATCTCGACTGCTGTG
Hs	MOG_Ex1_L	ChIP+RNA	154	ATCCCAAGAGGAAAGGAGGA
	MOG_Ex1_R			TGAAGGAGGAATGGATCAGG
Hs	SN5_L	ChIP+RNA	196	TCAGGGACCTCAAAGTGACC
	SN5_R			CAAGACACAGGCCAGAGACA
Hs	α-sat_L	ChIP+RNA	92	AACTCACAGAGTTGAACGATCC
	α-sat_R			ACCTCAAAGCGGCTGAAAT
Hs	βactin_E3E4_L	mRNA	327	GCGGGAAATCGTGCCTGACAT
	βactin_E3E4_R			GATGGAGTTGAAGGTAGTTTCGTG
Hs	βglobin_Ex2_L	ChIP+RNA	150	GCTGGTGGTCTACCCTTGGA
	βglobin_Ex2_R			AGGTTGTCCAGGTGAGCCAG
Mm	Aldoa_Ex1_L	ChIP+RNA	204	GTGGCTTTGAGCACAGATCA
	Aldoa_Ex1_R			GGGAAAGGAAAAAGCCAGAG
Mm	B1_L	ChIP+RNA	136	GTGGCGCACGCCTTAATC
	B1_R			GACAGGGTTTCTCTGTGTAG
Mm	GH_Ex1_L	ChIP+RNA	153	ACGCGCTGCTCAAAAATAT
	GH_Ex1_R			CACAGGAGAGTGCAGCAGAG
Mm	Hmbs_Ex1_L	ChIP+RNA	150	CCCATGTGCCTTCAGTCC
	Hmbs_Ex1_R			GTCACCGGCTCAGAACTCAC
Mm	HP_Ex1_L	ChIP+RNA	220	GTATGTCATGCTGCCTGTGG
	HP_Ex1_R			GTACCAGGTGTCTCCTCCA
Mm	Hprt_Ex1_L	ChIP+RNA	319	TTGTTGGATATGCCCTTGAC
	Hprt_Ex1_R			AAGCGACAATCTACCAGAGG
Mm	L1_L	ChIP+RNA	155	TTTGGGACACAATGAAAGCA
	L1_R			CTGCCGTCTACTCTCTTGG
Mm	Mj-sat_L	ChIP+RNA	162	CATGGAAAATGATAAAAACC
	Mj-sat_R			CATCTAATATGTTCTACAGTGTGG
Mm	Mn-sat_L	ChIP+RNA	74	GACGACTTGAAAAATGACGAAATC
	Mn-sat_R			CATATTCCAGGTCCTTCAGTGTGC
Mm	Mog_Ex1_L	ChIP+RNA	191	AATATCTGGCAAGGGTGACG
	Mog_Ex1_R			TATGGTTACCGGGGACAGAG
Mm	βactin_Ex1_L	ChIP+RNA	327	GCTACAGCTTCACCACCACA
	βactin_Ex1_R			ATGCCACAGGATTCCATACC
Mm	βactin_Ex2_L	mRNA	205	CTTTTCACGGTTGGCCTTAG
	βactin_Ex2_R			GGACTCCTATGTGGGTGACG

Hs: Homo sapiens
Mm: Mus Musculus

Table S.5

List of primers for chapter 4

species	primer name	purpose	product (bp)	primer sequence (5' to 3')
Mm	4930402E16Rik_L	mRNA	118	TGTGCCGGGCACTGGGCATA
	4930402E16Rik_R			TCTGCAGAGGGGTGTCCAGG
Mm	6820431F20Rik_L	mRNA	169	GGGAGTTCATGGCGGTGACGG
	6820431F20Rik_R			TGGCGGCCTCCCAGATGTGAC
Mm	Ankhd2_L	mRNA	113	CAGTCCTCCAGCAACAGTGA
	Ankhd2_R			GTGCAGGCATAAATGTGGTG
Mm	Ash1_L	mRNA	122	TAAGATGAGGGAGGCAATGG
	Ash1_R			GAGCAGCTGTAAGGGGTGAG
Mm	Atxn2_L	mRNA	106	CGCCTCAGACTGTTTTGGTA
	Atxn2_R			CAGCAGGACAACGACGAAG
Mm	Csnk2a1_L	mRNA	132	TGACCCCCATCTACCTTCTG
	Csnk2a1_R			AAATCCACAAGCAACGGTTC
Mm	Ddi2_L	mRNA	139	CCGTGTACTGTGTGCGGAGGG
	Ddi2_R			GCATAGACAATCTGGCTCTGGCG
Mm	Ddx6_L	mRNA	118	TTTCTTCCGGGCTGAGTAGA
	Ddx6_R			TGTGAGCCAGCGTAGTGTC
Mm	Dpysl3_L	mRNA	143	CTGTAATTCTGCGCCTCCTC
	Dpysl3_R			ATAAGGTTTCGGCACAGTGG
Mm	Eif4ebp2_L	mRNA	120	ACCTTACGTTGGGTGTCCTG
	Eif4ebp2_R			TAGCCTGTGCTGCTCTCTCA
Mm	Elf2_L	mRNA	102	TCAGGGAACGCACGATGGCA
	Elf2_R			GGCCCGTGTACCTCCCTCTT
Mm	Fryl_L	mRNA	181	CAGCCTAGGAAGCAGATTGG
	Fryl_R			GCACTGGGTGTCACAGAAGA
Mm	Hmbs_Ex1_L	mRNA	150	AGAAAAGTGCCGTGGGAAC
	Hmbs_Ex1_R			TGTTGAGGTTTCCCGAAT
Mm	HP1 β _L	mRNA	193	GGCTGAGGGGAGGGCCGTTA
	HP1 β _R			GGTGTGAAGGGTGACACTGCTCG
Mm	Hprt_L	mRNA	319	TTGTTGATATGCCCTTGAC
	Hprt_R			AAGCGACAATCTACCAGAGG
Mm	Itch_L	mRNA	157	GCTGTAGTCGGGGCTCGGGA
	Itch_R			AGCAAACCTGAAGTTCTCAATGGCT
Mm	Kif5b_L	mRNA	139	TGCTGCTTCCCAACACTAGA
	Kif5b_R			AGGAGCAGAGTCCCTCAACA
Mm	Kremen1_L	mRNA	200	TGCACGTACATTCAAATCCCATCG
	Kremen1_R			GGCACAGTGGGGTTTTAGTCACTC
Mm	Me2_L	mRNA	153	CGTGTGCATCGACGTGGGCA
	Me2_R			AGTGTGTTCCGGCCGTATCTGTCA
Mm	MLI3_L	mRNA	149	AGGGCCAATCATCAGTGAAC
	MLI3_R			GAGGGGCCTCTCTGTCTCT
Mm	Myh9_L	mRNA	121	GCTTCAAGTCTGCCTTCCAC
	Myh9_R			AAACACAGTTCAGGGCAAG
Mm	Nisch_L	mRNA	114	CACGTGAAGAAAGGCAGTCA
	Nisch_R			CATACCTGGCTTGGCTTGAT
Mm	Otub1_L	mRNA	122	CTTAGCCTCCCAAGGGTTTC
	Otub1_R			CCTTGAAGGTTGGCAGAGAG
Mm	Ptpre_L	mRNA	123	GCCTCCGTAAGACACAGAGC
	Ptpre_R			AACACCGGAGCTTCTTTGAA
Mm	Sf3b1_L	mRNA	120	CACAGCAGCACGCAAACTAT
	Sf3b1_R			TGGTGCTGGAAGTGTATGC
Mm	Sfi1_L	mRNA	196	GCGTACCTTTAATCCCAGCA
	Sfi1_R			TGATGCCTTCTCTGGTGTG
Mm	Sp1_L	mRNA	111	CTGGGAGGAGGAAAAAGACC
	Sp1_R			AAGGCTCAGCCATTGAGAAA
Mm	Spnb2_L	mRNA	161	TGACGACCACGGTAGCCACAGA
	Spnb2_R			AGCTTCACGCTCATCTGCCAGG
Mm	Sry_L	mRNA	120	GGGCCATGTCAAGCGCCCA
	Sry_R			CTGCATCCCAGCTGCTTGCTGA
Mm	β actin_Ex2_L	mRNA	205	CTTTTCACGGTTGGCCTTAG
	β actin_Ex2_R			GGACTCCTATGTGGGTGACG

Table S.6**List of genotyping primers**

primer name	mouse strain	primer sequence (5' to 3')
hFXN3_L	YG8 FRDA model	TTTGTC AACATGGGGTCTCA
hFXN3_R	YG8 FRDA model	CCAGGCAGAGCCTGAGTAAC
FXN-KO_WJ5	YG8 FRDA model	CTGTTTACCATGGCTGAGATCTC
FXN-wt_WN39	YG8 FRDA model	CCAAGGATATAACAGACACCATT
FXN-KO_WC76	YG8 FRDA model	CGCCTCCCCTACCCGGTAGAATTC
HP1 γ -common-L	HP1 γ -genetrap	GAGTGATTACCGACACCACCA
HP1 γ -wt-R	HP1 γ -genetrap	TTTAATCGGAGACTTGAAGAGC
HP1 γ -mut-L	HP1 γ -genetrap	GTTCGCTTCTCGCTTCTGTT
M31-AS51	HP1 β -overexp.	CTCTGCCCCCTTCTCATTCT
M31-AS143	HP1 β -overexp.	GGCCCCAACCTGACTG
YMTFP1	Four core genotype	CTGGAGCTCTACAGTGATGA
YMTRP1	Four core genotype	CAGTTACCAATCAACACATCA
hCD2_AS61	hCD2 transgenic	ATTGGCTTGTGAACATTTACCTA
hCD2_AS62	hCD2 transgenic	GAATGTCCAAGTTGATGTCCTG

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