

**INVESTIGATION INTO THE ROLE OF
HETEROCHROMATIN PROTEIN 1
GAMMA (HP1 γ) IN GENE REGULATION
IN MAMMALS**

LAW PUI PIK

Ph.D. Thesis

THE UNIVERSITY OF HONG KONG
&
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Abstract of thesis entitled

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Submitted by

LAW PUI PIK

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Heterochromatin protein 1 (HP1) was identified as a key component of the condensed DNA surrounding centromeres in most eukaryotic cells. Genes placed in close proximity to such constitutively dense heterochromatin regions were found to be stochastically silenced in a proportion of the cells leading to variegation of expression, a classical epigenetic phenomenon known as position effect variegation (PEV). Mutagenesis screens identified HP1 and SUV39H as powerful suppressors of variegation which were necessary for heterochromatin-mediated PEV. SUV39H was found to methylate the histone H3 tail at lysine 9 at heterochromatin providing a binding site for HP1. This discovery provided the first direct evidence for a histone or epigenetic code in which the ‘writer’ of the code on chromatin would be the SUV39H and the ‘reader’ HP1. This mechanism was subsequently found to be conserved from *S. pombe* to humans. A mechanism which shares features with PEV

has also been implicated in the pathogenesis of repeat-expansion diseases, such as Friedreich's ataxia, in which the affected genes are aberrantly silenced. In mice there are 3 highly conserved HP1 isoforms, HP1 α , HP1 β and HP1 γ . HP1 α and β are found by microscopy to be localised to constitutive heterochromatic condensed regions in the nucleus whereas HP1 γ has a pan-nuclear distribution and is therefore implicating in regulating euchromatic genes. There is a paucity of data examining how and where HP1 γ regulates gene expression *in vivo* at the genome-wide level. This thesis employed knockout and knockdown strategies to investigate this. In view of the early lethality of mice in which HP1 γ has been deleted by homologous recombination, the function of HP1 γ was studied here by establishing mouse embryonic fibroblast cell lines. Immunofluorescent microscopy revealed for the first time that HP1 γ was necessary for the localisation of HP1 β at constitutive heterochromatic regions in the nucleus in most cells. This delocalisation of HP1 β was associated with aberrant upregulation of the repetitive major satellite DNA associated with pericentromeric heterochromatin implying for the first time that HP1 γ plays an important role in maintaining centromeric heterochromatin in a silenced state thought to be important for the maintenance of genome integrity. Strikingly, analysis of the transcriptome revealed a large number of genes (4293) to be dysregulated in male cells compared to females (1186) where the effect of HP1 γ deficiency resulted in aberrant expression of immune related genes that would normally be repressed. This sexually dimorphic effect was investigated further by studying the effect of HP1 γ deficiency on the subset of 176 genes found to differ in expression between normal males and females. Moreover, HP1 γ in males was found essential for maintaining the relative repression of 114 genes in males compared to females, suggesting that the Y chromosome interacts with HP1 γ to reduce the expression of these genes. In summary, a novel function for HP1 γ in repressing pericentromeric DNA and in maintaining sexually dimorphic gene expression was discovered.

(473 words)

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A thesis submitted in partial fulfilment of the requirement for
the Degree of Doctor of Philosophy
at the University of Hong Kong and Imperial College London

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Declaration

I declare that this thesis represents my own work, except where due acknowledgement is made, and that it has not been previously included in a thesis, dissertation or report submitted to this University or to any other institution for a degree, diploma or other qualifications.

Signed.....

LAW PUI PIK

List of Publications

*Libri, V., C. Yandim, S. Athanasopoulos, N. Loyse, T. Natisvili, **P. P. Law**, P. K. Chan, T. Mohammad, M. Mauri, K. T. Tam, J. Leiper, S. Piper, A. Ramesh, M. H. Parkinson, L. Huson, P. Giunti and R. Festenstein* (2014). "Epigenetic and neurological effects and safety of high-dose nicotinamide in patients with Friedreich's ataxia: an exploratory, open-label, dose-escalation study." **Lancet 384(9942): 504-513.**

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Abbreviations

3C	Chromosome conformation capture
A	Adenine
A	Alanine
ac	Acetylation
<i>Adamtsl2</i>	A disintegrin and metalloproteinase with thrombospondin motifs-like 2
Ago2	Argonaute RISC catalytic component 2
AL-sat	Alpha satellite
ALT	Alternative lengthening of telomere
APB	ALT-associated PML nuclear bodies
APS	Ammonium persulphate
ATP	Adenosine triphosphate
ATRX	α -thalassemia/mental retardation syndrome protein
bp	Base pairs
BP	Biological processes
BARD1	BRCA1 associated RING domain 1
BCA	Bicinchoninic acid
BRCA1	Breast cancer 1, early onset
BSA	Bovine serum albumin
C	Cytosine
CaCl ₂	Calcium Chloride
CAF1	Chromatin assembly factor 1
CBX	Chromobox
<i>Cbx1</i>	Chromobox homolog 1
<i>Cbx3</i>	Chromobox homolog 3
<i>Cbx5</i>	Chromobox homolog 5
<i>Ccl8</i>	Chemokine (C-C motif) ligand 8
CD	Chromo domain
CD2	Cluster of differentiation 2
cDNA	Complementary deoxyribonucleic acid
CDS	Coding sequence
ChIP	Chromatin immunoprecipitation

ChIP-seq	Chromatin immunoprecipitation sequencing
CK2	Casein kinase II
Clr4	Histone H3 methyltransferase Clr4
cm	centimetre
CO ₂	Carbon dioxide
Co-IP	Co-immunoprecipitation
<i>Col26a1</i>	Collagen, type XXVI, alpha 1
CRISPR	Clustered regularly interspaced short palindromic repeats
Ct	Cycle threshold
CTCF	CCCTC-Binding Factor
CSD	Chromoshadow domain
CTE	Carboxyl terminal extension
DAPI	4',6-diamidino-2-phenylindole
DAXX	Death domain-associated protein
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNAse	Deoxyribonuclease
Dnmt1	DNA methyltransferase (cytosine-5) 1
Dnmt3a	DNA methyltransferase 3A
Dnmt3b	DNA methyltransferase 3B
dNTP	Deoxynucleotide
dsRNA	double stranded RNA
DTT	Dithiothreitol
E13.5	Embryonic day 13.5
E2F	E2F transcription factor
ECL	Enhanced chemiluminescence
EDTA	Ethylen diamin tetraacetatic acid
EF1 α	Elongation factor 1 alpha
EGFP	Enhanced Green Fluorescent Protein
EGTA	Ethylene glycol tetraacetic acid
EHMT	Euchromatin histone methyltransferase
ERBB	erb-b2 receptor tyrosine kinase
ERK	Extracellularly Regulated Kinases

ERV	Endogenous retroviral element
ESCs	Embryonic stem cells
E(var)	Enhancer of variegation
F	Female
FBS	Fetal bovine serum
<i>Fbxw10</i>	F-box and WD-40 domain protein 10
<i>FMR1</i>	Fragile X mental retardation 1 gene locus
FOXP3	Forkhead box P3
FRDA	Friedreich's ataxia
<i>FXN</i>	Frataxin gene
G	Guanine
g	Gravity
G9a	Euchromatic histone lysine N-methyltransferase 2
<i>Gapdh</i>	Glyceraldehyde 3-phosphate dehydrogenase
<i>Gda</i>	Guanine deaminase
GFP	Green fluorescent protein
GLP	Euchromatic histone methyltransferase 1
GO	Gene ontology
GOBPID	Gene ontology biological processes identity
<i>Gpr165</i>	G protein-coupled receptor 165
GTP	Guanosine triphosphate
GTPase	GTP monophosphatase
H	Histone
h	Human
H3f3a	H3 histone, family 3A
H3f3b	H3 histone, family 3B
HA	hemagglutinin
HBS	HEPES buffered saline
HDAC	Histone deacetylase
HDACi	Histone deacetylase inhibitor
HDM	Histone demethylase
HEK293T	Human Embryonic fibroblast 293T cells
HEPES	4-(2-Hydroxyethyl) piperazine-1-ethanesulfonic acid, N-(2-Hydroxyethyl) piperazine-N'-(2-ethanesulfonic

	acid)
HET	Heterozygous
<i>hFXN</i>	Human frataxin transgene
Hin	Hinge domain
<i>Hif3a</i>	Hypoxia inducible factor 3, alpha subunit
HIPK2	Homeodomain interacting protein kinase 2
HIRA	Histone regulator A
HIV	Human immunodeficiency virus
HKMT	Histone lysine methyltransferase
hMis12	MIS12 kinetochore complex component
<i>Hnrnp3</i>	Heterogeneous nuclear ribonucleoprotein H3
HOM	Homozygous
HP1	Heterochromatin protein 1
HRP	Horseradish peroxidase
HSP	Heat shock protein
ICR	Imprinted control region
IgG	Immunoglobulin G
<i>Igf2</i>	Insulin-like growth factor 2
IκKα	Inhibitor of kappa B kinase alpha
INCENP	Inner centromere protein antigens 135/155kDa
iPSC	Induced pluripotent stem cell
JmjC	Jumonji C
JNK	c-Jun N-terminal kinases
K	Lysine
KCl	Potassium Chloride
KD	Knockdown
kDa	Kilodalton
Kdm2a	Lysine (K)-specific demethylase 2A
Kdm3A	Lysine (K)-specific demethylase 3A
Kdm4A	Lysine (K)-specific demethylase 4A
Kdm4C	Lysine (K)-specific demethylase 4C
Kdm5d	Lysine (K)-specific demethylase 5D
KH ₂ PO ₄	Monopotassium phosphate
KO	Knockout

Ku70	X-ray repair complementing defective repair in Chinese hamster cells 6
L	Leucine
LBR	Lamin B receptor
LCRs	Locus control regions
M	Male
M	Molar
MAP	Mitogen activated protein
MAPK	Mitogen activated protein kinase
<i>Mdk</i>	Midkine
Me	Methylation
me1	Monomethylation
me2	Dimethylation
me3	Trimethylation
Med29	Mediator complex subunit 29
MEF	Mouse embryonic fibroblast
mESC	Mouse embryonic cell
mg	Milligram
MgCl ₂	Magnesium Chloride
MHC	Major histocompatibility complex
min	Minutes
ml	Millilitre
mm	Millimetre
mM	Millimolar
<i>Mmp13</i>	Matrix metalloproteinase 13
MMTV	Mouse mammary tumour virus
MNase	Micrococcal Nuclease
mRNA	Messenger ribonucleic acid
MW	Molecular weight
MYC	Myelocytomatosis oncogene
<i>Myl3</i>	Myosin, light polypeptide 3
NaCl	Sodium Chloride
<i>Nanog</i>	Nanog homeobox
NaOAc	Sodium Acetate

NaOH	Sodium hydroxide
Na ₂ PO ₄	Disodium phosphate
ncRNA	Non-coding RNA
<i>Neurod6</i>	Neurogenic differentiation 6
ng	Nano gram
NH ₄	Ammonium
nm	Nano metre
NPC	Neural progenitor cell
NTE	Amino terminal extension
<i>Nxpe5</i>	Neurexophilin and PC-esterase domain family, member 5
P	Proline
P	Phosphorylation
<i>Pappalysin 2</i>	Pappalysin 2
PAX3	Paired box 3
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PEI	Poly-ethyleneimine
PEV	Position effect variegation
pg	Pico gram
<i>Pgm2l1</i>	Phosphoglucomutase 2-like 1
PI	Propidium iodide
PIAS1	Protein Inhibitor of Activated STAT, 1
PIM1	Pim-1 proto-oncogene, serine/threonine kinase
PML-NB	Promyelocytic leukemia nuclear bodies
PMSF	Phenylmethysulfonyl fluoride
<i>Ppbbp</i>	Pro-platelet basic protein
<i>Ppp2r2b</i>	Protein phosphatase 2, regulatory subunit B, beta
PVDF	Polyvinylidene fluoride
qRT-PCR	Quantitative real time polymerase chain reaction
RIN	RNA integrity number
RNA	Ribonucleic acid
RNA-seq	Ribonucleic acid sequencing
RNAse	Ribonuclease

RNAse A	Ribonuclease A
RNAPII	RNA polymerase II
RPKM	Reads per kilobase of transcript per million mapped reads
rpm	Revolutions per minute
rRNA	ribosomal RNA
S	Serine
S phase	Synthesis phase
S5P-RNAPII	Serine-5 phosphorylated RNA polymerase II
S93P-HP1 γ	Serine 93 phosphorylated HP1 γ
SAM	S-adenosylmethionine
SD	Standard deviation
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
sec	Second
SEM	Standard error of the mean
SETDB1	SET domain, bifurcated 1
shRNA	Short hairpin ribonucleic acid
SIR	Silent information regulatory protein
SIRT1	Silent mating type information regulation 2 homolog 1 (Sirtuin1)
<i>Slc2a4</i>	Solute carrier family 2 (facilitated glucose transporter), member 4
SNP	single nucleotide polymorphism
snRNA	Small nuclear RNA
snRNP	Small nuclear ribonucleic proteins
<i>Spp1</i>	Secreted phosphoprotein 1
SRSF1	Serine/arginine-rich splicing factor 1
<i>Sry</i>	Sex determining region of chromosome Y
SUV39h	Suppressor of variegation 3-9 homologue
SUV4-20H2	Suppressor of variegation 4-20 homolog 2 (Drosophila)
Su(var)	Suppressor of variegation
T	Thymine

TAE	Tris/acetate/EDTA
TAFII130	TAF4 RNA polymerase II, TATA box binding protein (TBP)-associated factor
TBE	Tris/Borate/EDTA
TBS	Tris buffered saline
TE	Tris/EDTA
TEMED	Tetramethylethylenediamine
TES	Transcription end site
TFIID	Transcription factor II D
<i>Tgm2</i>	Transglutaminase 2, C polypeptide
TIF1 α	Transcription intermediary factor 1-alpha
TIF1 β	Transcription intermediary factor 1-beta
TIN2	Terf1 (TRF1)-interacting nuclear factor 2
TPE	Telomere position effect
Treg	Regulatory T cell
tRNA	Transfer RNA
TSDR	Treg-specific demethylation region
TSS	Transcription start site
U	Ubiquitination
U	Unit
UT	Untreated
UTR	Untranslated region
UV	Ultraviolet
V	Valine
V	Volt
v	Volume
w	Weight
WT	Wild type
μ g	Microgram
μ m	Micrometre
μ M	Micromolar
μ l	Microlitre
$^{\circ}$ C	Degree Celsius

Chapter 1 - Introduction

1.1 Epigenetics - an overview

‘Epigenetics’ describes the mechanisms that regulate gene expression and the inheritance of this information (transmission of memory of function) from one generation to the next, or through cell division from parental to daughter cells without a change in the sequence of DNA (Bird 2007). Thus, epigenetic regulation depends on modifications made to DNA or chromatin, the regulatory proteins that write, read and erase these modifications as well as the differential incorporation of variant histones into chromatin. The landscape of these epigenetic regulatory factors is referred to as the ‘epigenome’. A variety of cellular phenomena including gene activation and silencing, heterochromatin formation, position effect variegation, imprinting and X chromosome inactivation have been described to rely on epigenetic regulation. Abnormal changes in the epigenome have also been implicated in various diseases. In this thesis, different epigenetic factors and their roles in gene regulation will be described and discussed.

1.2 Chromatin

1.2.1 Chromatin structure

Genetic information of eukaryotic organisms is stored by DNA. In eukaryotic nuclei, genomic DNA is hierarchically packaged as chromatin. The fundamental unit of chromatin is a multifaceted and highly dynamic nucleoprotein complex known as the nucleosome (Kornberg 1974) which consists of an octamer of four core histones (2 copies each of H2A, H2B, H3 and H4) wrapped around by ~ 1.65 superhelical turns by 147 base pairs (bp) of DNA (Luger, Mader et al. 1997). The core histone proteins are composed of a globular domain and flexible ‘histone tails’ at the amino terminus which can be subjected to a diversity of post-translational modifications. Adjacent individual nucleosomes are connected by linker DNA and form ‘nucleosomal arrays’ also known as the ‘beads-on-a-string’ organisation (Figure 1.1) (Belmont, Dietzel et al. 1999, Horn and Peterson 2002). The length of linker DNA ranges between ~ 20 -90bp and varies among species, tissues or even within a single cellular genome (van Holde 1988). Linker DNA at the entry or exit sites of the nucleosome is associated with linker histones (e.g. H1 and H5) (Simpson 1978, Hamiche, Schultz et al. 1996, Syed, Goutte-Gattat et al. 2010) which influence the orientation of linker DNA and also organise the nucleosome arrays into the higher order 30nm chromatin fibre (Robinson, Fairall et al. 2006). Further compaction of the 30nm chromatin fibre by fibre-fibre interactions leads to the formation of the ~ 100 nm interphase chromosomes (Belmont, Braunfeld et al. 1989) and visible metaphase chromosomes of ~ 600 nm (Belmont, Dietzel et al. 1999). Factors that can influence the structure of chromatin include histone variants, histone modifications, DNA methylation and the binding of non-histone proteins (Li and Reinberg 2011) will be discussed in later sections (see section 1.3 and 1.4).

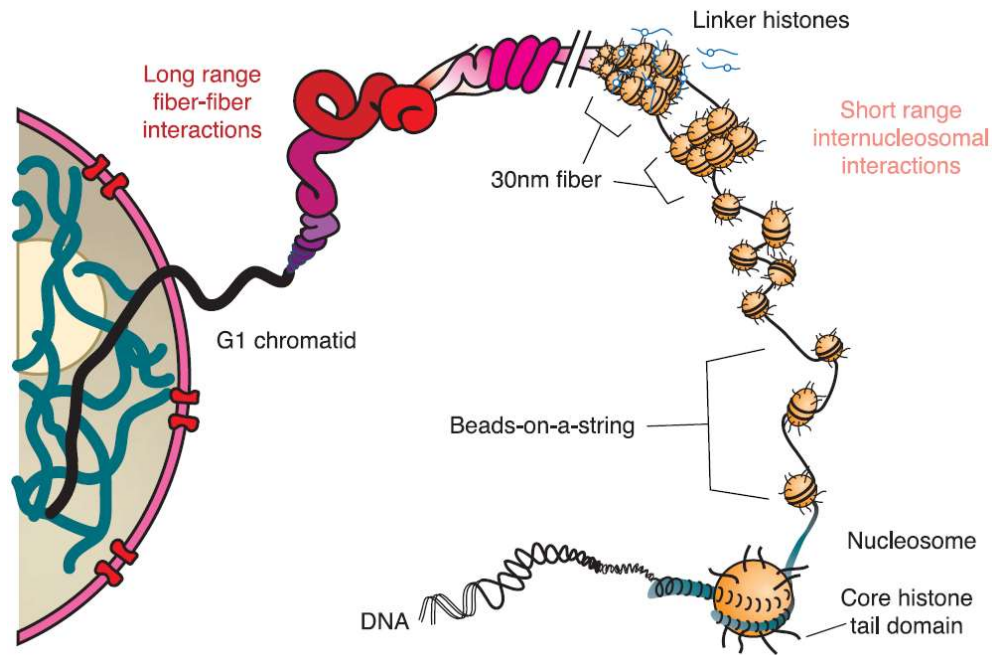


Figure 1.1 Schematic diagram of chromatin organisation. Genomic DNA is packaged by wrapping around the nucleosomes in the nucleus and forms a ‘beads-on-a-string’ organisation. Linker histones assist compaction of this structure which is thought to trigger formation of the 30nm fibre. By fibre-fibre interaction, the structure of chromatin is further compacted into heterochromatin. This figure is adapted and modified from Horn, P. J. and C. L. Peterson (2002). "Molecular biology. Chromatin higher order folding--wrapping up transcription." *Science* 297(5588): 1824-1827. Reprinted with permission from AAAS. Also with permission from Annual Review of Biophysics and Biomolecular Structure "Hansen, J. C. (2002). ‘Conformational dynamics of the chromatin fiber in solution: determinants, mechanisms, and functions.’ *Annu Rev Biophys Biomol Struct* 31: 361-392.”(Hansen 2002, Horn and Peterson 2002)

1.2.2 Euchromatin and heterochromatin

Chromatin of two distinct structures were first described by Emil Heitz almost nine decades ago on the basis of a series of cytogenetic observations that ‘heterochromatin’ in the nucleus remained condensed whereas ‘euchromatin’ underwent cycles of condensation and de-condensation throughout the cell cycle (Heitz, 1928). These two types of chromatin show distinct organisation and features (Figure 1.2) which contribute to the diverse influences on gene regulation.

Euchromatin, or the ‘true chromatin’ is characterised by its relatively open structure and the higher accessibility as measured experimentally by nucleases. It is replicated in early S-phase and rich in transcriptionally active genes. In contrast, heterochromatin is condensed and highly resistant to nucleases. It is gene poor and generally transcriptionally inactive. Heterochromatin is late replicating in S phase and rich in repetitive DNA sequences such as satellite repeats, transposable elements and retrovirus related repetitive sequences (Reviewed in - Dillon and Festenstein 2002, Grewal and Elgin 2002, Grewal and Moazed 2003). *Drosophila* nucleosomes in heterochromatin are regularly spaced with shorter linker DNA while nucleosome arrays in euchromatin are relatively disorganised (Wallrath and Elgin 1995, Sun, Cuaycong et al. 2001). Although consistently found surrounding structures such as centromeres and telomeres, smaller blocks of heterochromatin were also found to be interspersed throughout the chromosome (Reviewed in - Grewal and Moazed 2003). Moreover, the two types of chromatin are distinguished by a characteristic epigenetic profile. Euchromatin is characterised by hypomethylated DNA, abundant acetylation on histone H3/H4 and methylation of histone H3 lysine 4 (H3K4) (Reviewed in - Grewal and Jia 2007) whereas heterochromatin is distinguished by DNA methylation (Okano, Bell et al. 1999, Lehnertz, Ueda et al. 2003), hypoacetylation of histone (Moazed 2001) and enrichment of histone modifications such as methylation of histone 3 lysine 9 (H3K9), histone 3 lysine 27 (H3K27) and histone 4 lysine 20 (H4K20) (Peters, Kubicek et al. 2003, Schotta, Lachner et al. 2004, Ebert, Lein et al. 2006).

Heterochromatin can be further subdivided into constitutive and facultative heterochromatin. Constitutive heterochromatin which is composed predominately

of long stretches of repetitive DNA elements (such as alpha satellite (α -satellite) and γ -satellite variant II (γ -satellite II) (Lin, Sasi et al. 1993) in human and major satellite (Mj-sat) in mice (Mouse Genome Sequencing, Waterston et al. 2002)) is found in pericentromeric regions. Also, it remains visibly condensed during interphase of the cell cycle (Reviewed in - Dillon and Festenstein 2002). On the other hand, facultative heterochromatin is developmentally regulated and able to transit between heterochromatic and euchromatic states in response to cellular signals and gene activity (Reviewed in - Dillon and Festenstein 2002, Grewal and Jia 2007). One classical example of facultative heterochromatin includes the inactive X chromosome in female mammals which forms the condensed “Barr body” which is visible in interphase nuclei (Reviewed in - Dillon and Festenstein 2002, Dillon 2004). It is hypoacetylated and contains methylated histone H3K9me3 and H3K27me3 (Jeppesen and Turner 1993, Trojer and Reinberg 2007). It is also noteworthy that the two types of heterochromatin feature specific histone modifications whereby facultative heterochromatin is marked by high levels of H3K27me3 while constitutive heterochromatin is marked by H3K9me3 and H4K20me3 (Reviewed in - Trojer and Reinberg 2007).

Euchromatin

- Open structure
- Gene rich
- Rich in transcriptionally active genes
- Highly accessible for nucleases
- Early replicating in S-phase
- Disorganised nucleosome array
- Hypomethylated DNA
- Abundant histone H3/H4 acetylation
- Methylated on H3K4

Heterochromatin

- Highly condensed structure
- Gene poor
- Rich in repetitive DNA sequences
- Generally transcriptionally inactive
- Resistant to nucleases
- Late replicating in S phase
- Regular nucleosome array
- Enriched DNA methylation
- Hypoacetylation of histone H3/H4
- Methylated on H3K9, H3K27, H4K20

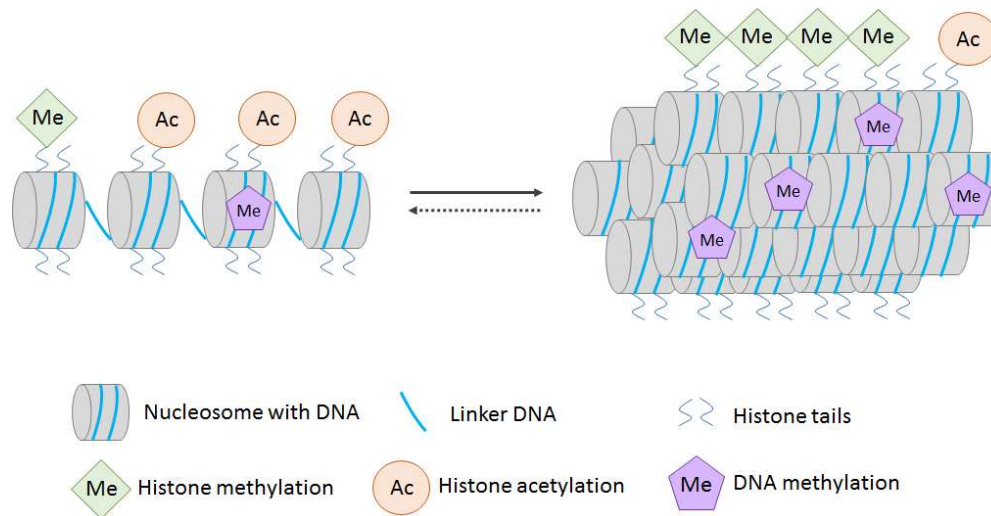


Figure 1.2 Schematic diagram demonstrating the organisation of euchromatin and heterochromatin. Euchromatin is characterised by its relatively open structure whereas heterochromatin is tightly compacted. Specific epigenetic profile features the two forms of chromatin. Key features of each form of the chromatin is summarised on the top of the diagram.

1.3 The epigenetic code

1.3.1 Overview

Intrinsic properties of nucleosomes are determined by distinct sets of epigenetic modifications including DNA methylation, histone post-translational modifications and the incorporation of histone variants. The combination of these modifications have been proposed to function as an ‘epigenetic code’ playing an important role in regulating nucleosome dynamics and function of underlying DNA (Jenuwein and Allis 2001). In this thesis, most attention will be paid to discussing how histone lysine methylation and histone variants affect the properties of chromatin and hence regulate gene activity.

1.3.2 Histone modifications

1.3.2.1 General introduction

The nucleosome, the fundamental unit of chromatin consists of an octamer of two molecules of each of the four core histone proteins. Each of the core histone proteins are composed of a globular domain and a flexible amino terminal ‘histone tail’ which protrudes from the nucleosome and allows a wide range of post-translational modifications. To date, at least eight different types of post-translational modifications (acetylation, methylation, phosphorylation, ubiquitylation, sumoylation, ADP ribosylation, deimination, proline isomerisation) have been identified over 60 different histone residues (Reviewed in - Kouzarides 2007). Figure 1.3 illustrates the common modifications of the core histones.

Histone modifications have at least two modes of function. Firstly, histone modifications affect the overall structure of chromatin directly by interfering with both inter-nucleosomal and histone/DNA interactions (Reviewed in - Berger 2002, Kouzarides 2007, Bannister and Kouzarides 2011). An important example of this includes the acetylation on the lysine residues on histones in which basic charges

of lysine are neutralised and the histone/DNA interactions are thereby weakened. Histone acetylation thus prevents the formation of higher-order chromatin structures and hence contributes positively to gene activities by increasing general accessibility of gene loci (Reviewed in - Kouzarides 2007, Bannister and Kouzarides 2011, Li and Reinberg 2011). On the other hand, histone modifications regulate binding of protein factors (positively or negatively) with specific regulatory or enzymatic activities to particular gene loci and hence influence processes such as transcription, DNA repair, replication and recombination (Reviewed in - Bannister and Kouzarides 2011) and alternative splicing (Luco, Pan et al. 2010). The ‘writers’, ‘readers’ and ‘erasers’ of different histone modifications have been identified and characterised.

It is also noteworthy that modifications can occur sequentially or in combination on one or more histone tails within a nucleosome. In fact, many different histone modifications have been found to be synergistic or antagonistic and hence have led to the hypothesis of a ‘histone code’, which is defined as the combinations of histone modifications that contribute to a unique cellular response (Reviewed in - Strahl and Allis 2000). This ‘crosstalk’ between histone modifications adds an extra level of complexity in gene regulation and has been suggested to fine-tune the overall control (Reviewed in - Bannister and Kouzarides 2011).



Figure 1.3 Schematic diagram showing the main post-translational modifications on core histone proteins. Amino acid sequences of each core histone protein were shown in grey and the numbers on the bottom indicate the amino acid positions. Post-translational modifications (acetylation (A) (blue), methylation (M) (red), phosphorylation (P) (yellow) and ubiquitination (U) (green)) are shown on top of the amino acid sequences. This figure is adapted by permission from Macmillan Publishers Ltd: Nature Biotechnology “Portela, A. and M. Esteller (2010). ‘Epigenetic modifications and human disease.’ *Nat Biotechnol* 28(10): 1057-1068”, copyright (2010) (Portela and Esteller 2010)

1.3.2.2 Histone lysine methylation

To date, seven different lysine residues on histone H3 and H4 have been identified as methylation sites. Histone lysine residues can harbor one (mono-methylation), two (di-methylation) or three (tri-methylation) methyl groups on their amino groups. These different levels of modification state can potentially lead to diverse biological outcomes through different ‘readers’ that recognise these marks and hence add an extra level of complexity to gene regulation. Unlike acetylation, methylation of histone does not alter the charge of histone proteins and thus is considered to regulate activity of chromatin by recruiting other effector proteins for downstream functions. Rather than largely correlating with gene activation like acetylation, methylation of histone lysine exerts both gene activation and repression depending on its target lysine residues (Reviewed in - Martin and Zhang 2005, Ng, Yue et al. 2009, Bannister and Kouzarides 2011). Histone lysine residues with different level of methylation state showed similar or distinct genomic distributions and functions (Barski, Cuddapah et al. 2007). In general, methylation of H3K9, H3K27 and H4K20 are regarded as the repression marks while methylation of H3K4, H3K36 and H3K79 are regarded as the activation marks (Reviewed in - Kouzarides 2007). More recently, studies revealed H3K56 as a methylation site and implicated it in DNA replication (H3K56me) and gene silencing (H3K56me3) (Yu, Song et al. 2012, Jack, Bussemer et al. 2013). Table 1.1 summarises the different histone lysine methylation and their well characterized functions.

Histone lysine methylation	Methylation state	Role in gene regulation	Genomic distribution	References
H3K4	H3K4me1	Gene activation	Enhancer, surrounding TSS	1, 2, 3
	H3K4me2	Gene activation	Surrounding TSS	1, 2, 3
	H3K4me3	Gene activation	TSS	1, 2, 3
H3K9	H3K9me1	nd	Surrounding TSS	1
	H3K9me2	Gene repression	Broad distribution	1
	H3K9me3	Gene repression	Broad distribution	3, 4, 5, 6
H3K27	H3K27me1	Gene activation	Surrounding TSS, enhancer	1, 7, 8
	H3K27me2	Enhancer regulation	Broad distribution	1, 8
	H3K27me3	Gene repression	Broad distribution	1, 9, 10
H3K36	H3K36me1	nd	Broad distribution	1
	H3K36me2	DNA repair	nd	11
	H3K36me3	Gene activation	Transcribed region	1, 12, 13
H3K56	H3K56me1	DNA replication	nd	14
	H3K56me3	Gene repression	nd	15
H3K79	H3K79me1	nd	Broad distribution	1, 16
	H3K79me2	nd	Broad distribution	1, 16
	H3K79me3	Gene activation	Promoter	1, 16
H4K20	H4K20me1	Gene activation	Downstream of TSS	1, 12, 17
	H4K20me2	DNA damage response	nd	18
	H4K20me3	Gene repression	Broad distribution	1, 5

Table 1.1 Summary of the associated functions and the general genomic distribution of the common histone lysine methylation in mammals. TSS, transcription start site; nd, not determined. TSS marks are generally sharply defined whereas other modifications have a broader distribution along genes or regulatory elements as indicated in the table. References used are: (Barski, Cuddapah et al. 2007)¹, (Deb, Kar et al. 2014)², (Shen, Shulha et al. 2014)³, (Bannister, Zegerman et al. 2001)⁴, (Schotta, Lachner et al. 2004)⁵, (Kimura 2013)⁶, (Steiner, Schulz et al. 2011)⁷, (Ferrari, Scelfo et al. 2014)⁸, (Boyer, Plath et al. 2006)⁹, (Lee, Jenner et al. 2006)¹⁰, (Fnu, Williamson et al. 2011)¹¹, (Vakoc, Sachdeva et al. 2006)¹², (Wagner and Carpenter 2012)¹³, (Yu, Song et al. 2012)¹⁴, (Jack, Bussemer et al. 2013)¹⁵, (Kim, Choi et al. 2014)¹⁶, (Talas, Lindner et al. 2005)¹⁷, (Jorgensen, Schotta et al. 2013)¹⁸

1.3.2.2.1 Methylation of histone H3 lysine 9 (H3K9)

Methylation of histone H3 lysine 9 (H3K9) is typically associated with silent chromatin domains including constitutive heterochromatin (pericentric and telomeric regions). It has been found in silent chromatin domains from yeasts to mammals and is regarded as the hallmark of heterochromatin (Jenuwein and Allis 2001, Peters, Kubicek et al. 2003). However, H3K9 methylation of different levels was actually found in different chromatin compartments. In mammals, trimethylated H3K9 is enriched in pericentric heterochromatin and associated preferentially with silent genes (Bannister, Zegerman et al. 2001). Although dimethylated H3K9 also shows preference for silent genes, it is more often found in euchromatic regions. Interestingly, the relatively less characterised monomethylated H3K9 is enriched in euchromatic regions and concentrated in promoter regions surrounding the transcriptional start site of actively transcribed genes. This observation led to the hypothesis that mono-methylated H3K9 may be implicated in transcriptional activation (Peters, Kubicek et al. 2003, Rice, Briggs et al. 2003, Barski, Cuddapah et al. 2007). Indeed, a growing number of studies revealed association of H3K9 methylation on transcriptionally active genes (Vakoc, Mandat et al. 2005, Vakoc, Sachdeva et al. 2006, Barski, Cuddapah et al. 2007, Kim, Kiefer et al. 2007) implicating it in other regulatory processes including alternative splicing (Saint-Andre, Batsche et al. 2011, Ameyar-Zazoua, Rachez et al. 2012, Yearim, Gelfman et al. 2015) and transcription termination (Skourti-Stathaki, Kamieniarz-Gdula et al. 2014). These findings suggested that the role of H3K9 methylation is not solely silencing as was originally shown.

The ‘writers’ of histone lysine methylation are called histone lysine methyltransferases (HKMT) which catalyse the transfer of a methyl group from S-adenosylmethionine (SAM) to the ϵ -amino group of the histone lysine residues. Interestingly, all of the HKMTs identified contain a SET domain which harbors the enzymatic activity, except the H3K79 methyltransferase Dot1 enzyme whose enzymatic activity come from its catalytic methylase fold (Min, Feng et al. 2003).

In mammals, a number of HKMTs responsible for methylation of H3K9 have been identified. SUV39H1 was the first H3K9 HKMT identified in mammals (Rea,

Eisenhaber et al. 2000). SUV39H (suppressor of variegation 3-9 homolog 1 (*Drosophila*)) has two isoforms: SUV39H1 and SUV39H2 (Peters, O'Carroll et al. 2001) which are highly evolutionarily conserved from yeast to human (yeast: Clr4; *Drosophila*: Su(var)3-9; mouse: Suv39h; human: SUV39H). Silencing activities of SUV39H are associated with constitutive heterochromatin (Peters, O'Carroll et al. 2001, Rice, Briggs et al. 2003) as well as euchromatic genes (Nielsen, Schneider et al. 2001, Ait-Si-Ali, Guasconi et al. 2004, Duong and Weitz 2014, Zhang, Liu et al. 2014) and retrotransposon elements (Bulut-Karslioglu, De La Rosa-Velazquez et al. 2014). SUV39H is responsible for tri-methylation of H3K9 (H3K9me3) and provides the binding site for the 'reader' protein heterochromatin protein 1 (HP1) (Bannister, Zegerman et al. 2001, Lachner, O'Carroll et al. 2001). HP1 is a non-histone chromosomal protein that binds H3K9me3 with its chromodomain and with its chromoshadow domain interacts with a wide range of effector proteins (Reviewed in - Hiragami and Festenstein 2005, Kwon and Workman 2011) and thereby contributes to downstream functions. Binding of HP1 to H3K9me3 is also likely to be involved in stabilisation of higher order chromatin structure (Bannister, Zegerman et al. 2001, Lachner, O'Carroll et al. 2001, Nakayama, Rice et al. 2001, Canzio, Liao et al. 2013, Nozawa, Nagao et al. 2013). More details on the function of HP1 can be found in section 1.4.

G9a and GLP are another two HKMTs that catalyse mono- and di- methylation of H3K9 in mammals. They are closely related paralogs (Dillon, Zhang et al. 2005) and have been regarded as the euchromatin histone methyltransferases (EHMTs) due to their specificity in methylating H3K9 residues in euchromatic regions but not in pericentric heterochromatin (Tachibana, Sugimoto et al. 2001, Tachibana, Sugimoto et al. 2002, Peters, Kubicek et al. 2003, Rice, Briggs et al. 2003, Tachibana, Ueda et al. 2005). G9a and GLP were initially shown to function as a heterodimeric complex (Tachibana, Sugimoto et al. 2002). Subsequent studies suggested that the formation of their heterodimer may occur in a tissue- and development stage-dependent manner (Reviewed in - Kramer 2015) and it has been shown that they can also function independently (Tachibana, Matsumura et al. 2008, Liu, Zhang et al. 2015). As well as catalysing the methylation of histone lysine residues, G9a and GLP also methylate themselves (Sampath, Marazzi et al. 2007, Chang, Sun et al. 2011) and a number of non-histone proteins (Herz, Garruss et al.

2013, Lanouette, Mongeon et al. 2014, Biggar and Li 2015). Intriguingly, G9a and GLP have been shown to activate transcription independent of their methyltransferase activities (Lee, Northrop et al. 2006, Bittencourt, Wu et al. 2012, Chaturvedi, Somasundaram et al. 2012, Ohno, Shinoda et al. 2013, Wang, Xu et al. 2013).

The third H3K9-specific HKMT is SETDB1 (SET domain, bifurcated 1), which is also known as ESET. SETDB1 is specific for catalysing di- or tri-methylation of H3K9 (Schultz, Ayyanathan et al. 2002, Yang, Xia et al. 2002). It is also able to convert di-methylated H3K9 to tri-methylated H3K9 with the help of mAM ATPase which enhances the catalytic activity of SETDB1 (Wang, An et al. 2003). Although SETDB1 silencing activities have been shown to associate mainly with euchromatin (Schultz, Ayyanathan et al. 2002, Yang, Xia et al. 2002, Kim, Geng et al. 2003, Carling, Kim et al. 2004, Gazzero, Abbondanza et al. 2006, Li, Rauch et al. 2006), a more recent study showed that it is also responsible for repressing major satellite and endogenous retroviral elements in mammals (Matsui, Leung et al. 2010).

It is important to note that methylation of lysine is a reversible process. ‘Erasers’ of histone methylation i.e. the histone demethylases (HDMs) have been identified. Of particular interest, H3K9 residues are demethylated by the Jumonji C (JmjC) domain demethylases. KDM3A (JHDM2A) has been shown to demethylate mono- and di-methylated H3K9 while KDM4A (JHDM3A) and KDM4C (JMJD2C) have been shown to demethylate tri-methylated H3K9 (Fodor, Kubicek et al. 2006, Klose, Yamane et al. 2006, Whetstine, Nottke et al. 2006). These HDMs were shown to associate with transcriptional activation (Yamane, Toumazou et al. 2006, Ishimura, Terashima et al. 2009) and a growing number of studies implicate these enzymes in tumorigenesis (Cloos, Christensen et al. 2006, Ishimura, Terashima et al. 2009, Luo, Chang et al. 2012, Black, Manning et al. 2013, Li, Xue et al. 2014, Mahajan, Lawrence et al. 2014, Parrish, Sechler et al. 2015).

1.3.3 Histone variants

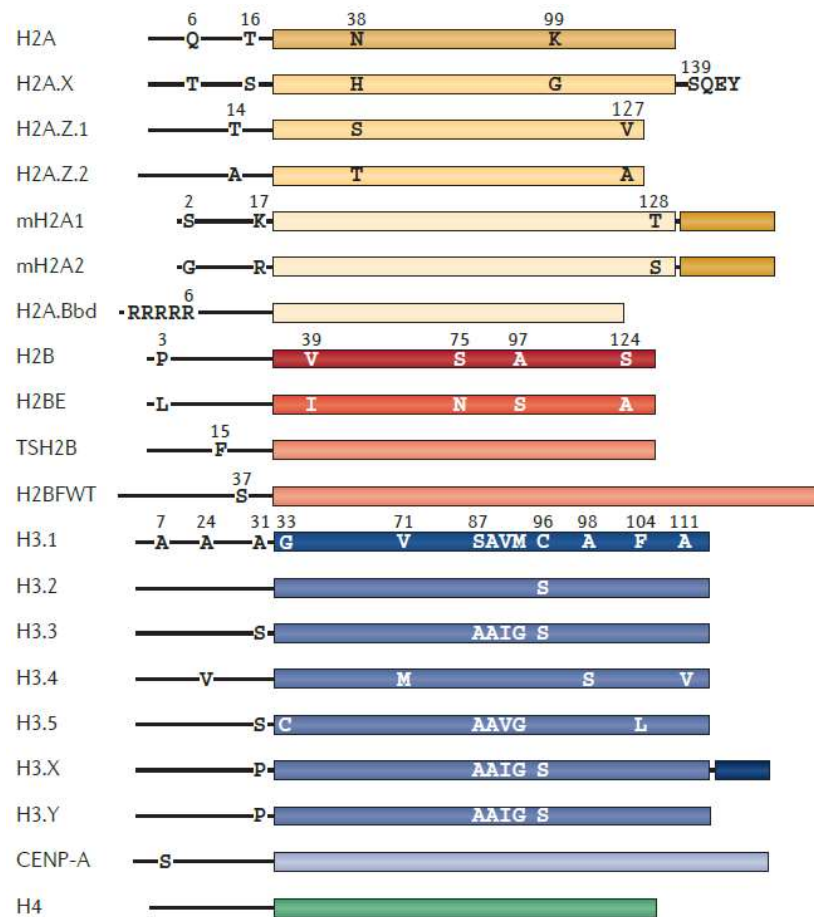
1.3.3.1 Overview

‘Histone turnover’ is a mechanism which refers to the exchange of subunits of the nucleosome or entire nucleosomes with newly synthesised histones or other protein components. This swapping mechanism contributes to the dynamic nature of chromatin and allows accessibility of factors to DNA to be regulated. These factors are involved in processes such as transcription, recombination, repair and replication of DNA. Thus, histone turnover regulates chromatin structure and functions by promoting (with increased turnover rate) or restricting (with decreased turnover rate) the binding of factors such as the transcription machinery (Reviewed in - Venkatesh and Workman 2015). It is noteworthy that euchromatin, which has a relatively high histone turnover rate, is highly accessible to gene regulatory factors while heterochromatin, which has a lower histone turnover rate, is generally inaccessible (Dion, Kaplan et al. 2007, Jamai, Imoberdorf et al. 2007, Rufiange, Jacques et al. 2007, Deal, Henikoff et al. 2010). Moreover, incorporation of different histone variants plays a key role in altering chromatin properties as each variant has a different turnover rate and also contributes to the different chemical and physical properties of nucleosomes. During transcription, histone turnover can be regulated co-operatively or individually by factors such as chromatin modifiers, chromatin remodelers and histone chaperones (Reviewed in - Venkatesh and Workman 2015). In this section, I will firstly introduce the different types of histone variants identified in mammals and compare the differences between canonical H3 and H3.3 on their properties. The role of mammalian H3.3 in gene regulation will also be reviewed.

1.3.3.2 Introduction of histone variants

To date, variant form of histones have been described for all histones except histone H4. In mammals, eleven forms of H1 (H1.0, H1.1, H1.2, H1.3, H1.4, H1.5, H1t, H1T2, HILS1, H1oo and H1x), seven forms of H2A (H2A, H2A.X, H2A.Z.1, H2A.Z.2, macroH2A1, macroH2A2 and H2A.Bbd), four forms of H2B (H2B, H2BE, TSH2B and H2BFWT) and eight forms of H3 (H3.1, H3.2, H3.3, H3.4, H3.5, H3.X, H3.Y and CENP-A) have been identified (Reviewed in - Maze, Noh et al. 2014, Turinetto and Giachino 2015, Venkatesh and Workman 2015). Variant histones are classified into ‘canonical’ or ‘non-canonical’ histones. Canonical histones are expressed exclusively during S-phase and incorporated into chromatin in a DNA replication-dependent manner (Marzluff, Gongidi et al. 2002). On the other hand, non-canonical histones, also known as ‘replacement histones’ are expressed throughout the cell cycle and their incorporation is independent of DNA replication (Ahmad and Henikoff 2002). Expression of replacement histones can be somatic or germline-specific and they may be localised at specific chromatin compartments (Reviewed in - Venkatesh and Workman 2015). Replacement histones usually differ from the canonical histones by a few amino acids substitution or with an additional domain. For example, histone variant H3.3 differs from canonical H3.1 by five amino acid differences while histone variant macroH2A has an extra macro domain in its carboxyl terminal tail when compared to canonical H2A (Reviewed in - Biterge and Schneider 2014). Figure 1.4 summarises the structural and amino acid sequence differences between different histone variants.

A



B

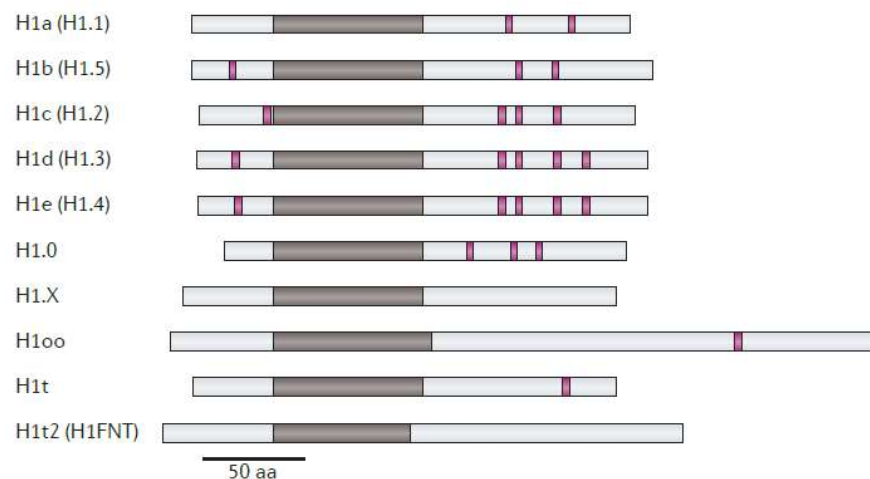


Figure 1.4 Schematic diagram summarising the structural and amino acid sequence differences between human canonical or variant core histones (A) and linker histone (B). (A) Each box and line structure represents the linearised structure of each histone variants. Different families of histone proteins are indicated by different colours (H2A (yellow); H2B (red); H3 (blue) and H4 (green)). Black lines represent

the unstructured amino terminal domains of histone proteins. Amino acid sequence differences and their positions are indicated by the letter and the number above. (B) Linearised structure of linker histone H1 variants are shown. Globular domains are indicated by dark grey boxes while the unstructured amino and carboxyl terminal domains are indicated by light grey boxes. Purple boxes represent the canonical serine/threonine PXX phosphorylation sites. Due to high degree of sequence divergence between the different H1 variants, amino acid sequence differences are not shown here. aa, amino acid; mH2A1, macroH2A. This figure is adapted by permission from Macmillan Publishers Ltd: Nature Review Genetics “Maze, I., K. M. Noh, A. A. Soshnev and C. D. Allis (2014). ‘Every amino acid matters: essential contributions of histone variants to mammalian development and disease.’ *Nat Rev Genet* 15(4): 259-271.”, copyright (2014) (Maze, Noh et al. 2014).

1.3.3.3 Canonical histone H3 vs. replacement histone H3.3

As mentioned previously, mammalian histone H3 has eight variants (H3.1, H3.2, H3.3, H3.4, H3.5, H3.X, H3.Y and CENP-A) in which H3.1 and H3.2 are the canonical histones while the others are referred to as replacement histones. These variants are considered to have distinct functions in organising chromatin structure. Canonical histone H3.1 and H3.2 (generally referred to as histone H3) and the replacement histone H3.3 are the most abundant H3 variants which are found in all types of tissues and cells. These three variants are implicated in regulation of chromatin structure and transcription (Reviewed in - Weber and Henikoff 2014). Histone H3.4 and H3.5 are germline-specific variants that can be found in testis and sperm (Albig, Ebentheuer et al. 1996, Witt, Albig et al. 1996, Schenk, Jenke et al. 2011) while H3.X and H3.Y have recently been suggested to regulate cellular responses to extracellular stimuli (Wiedemann, Mildner et al. 2010). Lastly, CENP-A is specifically localised to the centromere and serves as an epigenetic marker for the kinetochore formation sites (Palmer, O'Day et al. 1987).

Although canonical H3 and variant H3.3 are both implicated in regulation of chromatin structure and gene regulation, they show different features in various aspects. Firstly, the genes encoding canonical H3 and variant H3.3 have different genomic organisation. Genes encoding canonical histones H3.1 and H3.2 are intron-less and clustered in tandem repeat arrays. The mRNAs of these genes are not polyadenylated, instead they end with a stem loop structure followed by a

stretch of purine-rich sequence that is complementary to the 5' end of the U7 snRNA (Marzluff, Gongidi et al. 2002). This special genomic organisation allows bulk expression of canonical histone during S-phase and ensures sufficient supply of histone proteins for their incorporation into the chromatin in a DNA replication-dependent manner (Osley 1991). In contrast, genes encoding replacement histone H3.3 (*H3f3a* and *H3f3b*, also known as H3.3A and H3.3B) are found singly in the genome. These genes contain introns and their mRNAs are polyadenylated. Although having different expression patterns and distinct untranslated regions, *H3f3a* and *H3f3b* encode the identical H3.3 protein (Bramlage, Kosciessa et al. 1997, Frank, Doenecke et al. 2003). *H3f3a* and *H3f3b* are expressed throughout the cell cycle and can be incorporated during or outside S-phase of the cell cycle, independently from DNA replication (Ahmad and Henikoff 2002).

Histone H3.3 protein differs from the canonical histone H3.1 and H3.2 by five and four amino acids respectively (Figure 1.5). The alanine (A) residue at position 31 in the amino-terminal tail of histone H3.1 and H3.2 is replaced by a serine (S) residue in H3.3 which can be phosphorylated during mitosis (Hake, Garcia et al. 2005). Other amino acid differences can be found at residues 87, 89, 90 and 96 which are located at the globular domain of histone H3. Amino acid differences between the variants do not lead to any structural changes of the nucleosome (Tachiwana, Osakabe et al. 2011), however, they are crucial for binding of the histone chaperones (Elsasser, Huang et al. 2012, Liu, Xiong et al. 2012), the genomic localisation of the histone proteins (Goldberg, Banaszynski et al. 2010) and have been proposed to regulate the stability of histone-histone interaction (Reviewed in - Hake and Allis 2006).

H3 variants are deposited into the genome via different pathways which are tightly regulated by distinct histone chaperone proteins (Ray-Gallet, Woolfe et al. 2011). Incorporation of canonical histone H3 is mediated by chromatin assembly factor 1 (CAF1) during DNA replication (Tagami, Ray-Gallet et al. 2004) and DNA repair (Polo, Roche et al. 2006). Deposition of H3.3 in actively transcribed regions is mediated by the histone regulator A (HIRA) complex independent of DNA synthesis (Tagami, Ray-Gallet et al. 2004, Goldberg, Banaszynski et al. 2010) while death domain-associated protein (DAXX) together with the chromatin remodeler

α -thalassemia/mental retardation syndrome protein (ATR-X) deposits H3.3 at telomeric and pericentric heterochromatin (Drane, Ouararhni et al. 2010, Goldberg, Banaszynski et al. 2010, Lewis, Elsaesser et al. 2010). Furthermore, H3.3 deposition at promyelocytic leukemia nuclear bodies (PML-NBs) has recently been shown and its implication in heterochromatin organisation has been suggested (Corpet, Olbrich et al. 2014).

DNA replication-independent incorporation of replacement histone H3.3 requires displacement of existing nucleosomes for its deposition and hence contributes to the dynamics of chromatin. Turnover of H3.3 has been suggested to maintain the transient exposure of certain genomic regions and hence the access of different transcription factors (Reviewed in - Henikoff 2008). H3.3 turnover occurs in genomic regions including promoters, enhancers, gene bodies and heterochromatic regions. Interestingly, H3.3 turnover rate varies in different genomic regions: it is high at active promoters and enhancers, intermediate in gene bodies and low at inactive promoters and heterochromatic regions (Huang, Zhang et al. 2013, Kraushaar, Jin et al. 2013, Ha, Kraushaar et al. 2014).

Finally, canonical histone H3 and histone variant H3.3 are enriched for different sets of post-translational modifications. The histone variant H3.3-containing nucleosome is enriched in active chromatin marks such as H4 acetylation, methylation on H3K4 and H3K36 and acetylation on H3K9 and H3K14 (McKittrick, Gafken et al. 2004, Hake, Garcia et al. 2006, Loyola, Bonaldi et al. 2006). In contrast, canonical histone H3.2 is enriched in silencing marks such as H3K27 methylation which is associated with facultative heterochromatin while H3.1 is associated with both active and inactive marks (Hake, Garcia et al. 2006). Also, Loyola *et al* showed that H3K9me1 is found on non-nucleosomal histone H3 thereby priming it for future methylation by SUV39H (H3K9me3 HMT). Interestingly, H3K9me1 is enriched on non-nucleosomal H3.1 when compared to H3.3. In addition, HP1 α , which is found predominantly at heterochromatin (Minc, Allory et al. 1999), was found to preferentially associate with H3.1 containing purified oligonucleosomes whereas HP1 γ , which is found in both euchromatic and heterochromatic regions (Minc, Courvalin et al. 2000), associated with both H3.1 and H3.3 oligonucleosomes (Loyola, Bonaldi et al. 2006). This observation further

supports the connection between H3.3 and active transcription. Although enriched with active histone marks, H3.3 also harbors silencing marks and plays important roles in the genome. A recent study showed that H3.3 dependent H3K9me3 is involved in silencing endogenous retroviral elements (ERV) in mouse embryonic stem cells (mESCs) (Elsasser, Noh et al. 2015). Furthermore, in mESCs, H3.3 has also been shown to target H3K9me3 to the telomere and repress telomeric repeat (TERRA) expression as well as maintaining telomere integrity (Udugama, Chang et al. 2015).

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H3.1      MARTKQTARKSTGGKAPRKQLATKAARKSAFATGGVKKPHRYRPGTVALREIRRYQKSTE 60
H3.2      MARTKQTARKSTGGKAPRKQLATKAARKSAFATGGVKKPHRYRPGTVALREIRRYQKSTE 60
H3.3      MARTKQTARKSTGGKAPRKQLATKAARKSAFSTGGVKKPHRYRPGTVALREIRRYQKSTE 60
          *****:*****

H3.1      LLIRKLPFQRLVREIAQDFKTDLRFQSSAVMALQEACEAYLVGLFEDTNLCAIHAKRVTI 120
H3.2      LLIRKLPFQRLVREIAQDFKTDLRFQSSAVMALQEASEAYLVGLFEDTNLCAIHAKRVTI 120
H3.3      LLIRKLPFQRLVREIAQDFKTDLRFQSAATGALQEASEAYLVGLFEDTNLCAIHAKRVTI 120
          *****:*****

H3.1      MPKDIQLARRIRGERA 136
H3.2      MPKDIQLARRIRGERA 136
H3.3      MPKDIQLARRIRGERA 136
          *****

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Figure 1.5 Sequence alignment of mouse histone H3 variants H3.1, H3.2 and H3.3. Differences in amino acids were highlighted by orange boxes and their position is indicated by the number above. Canonical histone H3.1 and H3.2 are very similar to each other with only one amino acid difference at position 96. H3.1 and H3.2 differ from the replacement histone H3.3 by 5 and 4 amino acids respectively at position 31 (located at the N terminal tail), 87, 89 and 90 (located at the globular domain). Sequence alignment was generated by the online alignment tool ClustalW2 (powered by EMBL-EBI).

1.3.3.4 Distribution and functions of histone variant H3.3

Genome-wide distribution of H3.3 in mammals has been revealed recently by a number of studies using chromatin immunoprecipitation followed by high-throughput sequencing (ChIP-seq). These studies showed localisation of H3.3 to elements located in both euchromatic (e.g. promoters, enhancers, gene bodies) and heterochromatic regions (e.g. telomeric regions, interspersed repeats) and displays important regulatory functions (Goldberg, Banaszynski et al. 2010, Wen, Li et al. 2014, Elsasser, Noh et al. 2015, Voon, Hughes et al. 2015). Indeed, functions of H3.3 in different mammalian models have been studied extensively in recent years. Some examples of recent studies on the function of H3.3 are provided here.

1.3.3.4.1 Active role of H3.3 in gene regulation

In HeLa cells, H3.3 was implicated in activation of the heat shock induced expression of HSP70 genes. Of particular interest, this was found to operate coordinately with HP1 γ in which binding of these proteins to the HSP70 genes was found to be interdependent and correlated with the level of HSP70 expression (Kim, Heo et al. 2011).

In mESCs, enrichment of H3.3 around the transcription start site (TSS) of bivalent genes was maintained when these genes become transcriptionally active during mESC differentiation. H3.3 is also incorporated into the gene body in correlation with markers of transcription: histone marks H3K36me₃, H3K4me₃ and H3k4me₃ as well as serine-5 phosphorylated RNA polymerase II (S5P-RNAPII). However, bivalent genes that remained silent showed reduced H3.3 at the TSS upon differentiation (Goldberg, Banaszynski et al. 2010).

1.3.3.4.2 Repressive role of H3.3 in gene regulation

A number of recent studies implicate H3.3 in regulation of telomere function. Udugama and colleagues showed that complete knockout of H3.3 led to a reduction

in silencing histone marks H3K9me3, H4K20me3 and also the telomere-targeting H3.3 chaperone ATRX at telomeres (Udugama, Chang et al. 2015). Also, it has been shown in another study that knockdown of ATRX significantly reduced HP1 α at the telomeres (Wong, McGhie et al. 2010). Both of these studies showed an associated telomere dysfunction phenotype with depletion of H3.3 at telomere. Moreover, H3.3 at telomeres has been shown to repress telomeric RNA expression (Goldberg, Banaszynski et al. 2010, Udugama, Chang et al. 2015). Taken together, these studies suggest that H3.3 is important in maintaining heterochromatin formation at the telomere and hence its integrity.

Banaszynski *et al* implicated H3.3 in establishing silencing histone H3K27me3 at the promoters of developmentally regulated genes in mESCs. Depletion of H3.3 also led to dysregulation of bivalent genes upon differentiation and hence altered the developmental potential of mESCs. This study suggests that chromatin dynamics driven by H3.3 is important in establishing a proper chromatin landscape to allow appropriate regulation of genes to developmental cues (Banaszynski, Wen et al. 2013).

As mentioned in section 1.3.3.3, H3.3 has been shown to regulate ERVs in mESCs. Elsässer *et al* showed that ATRX/DAXX-mediated H3.3 is important for H3K9me3 on ERVs. Deletion of H3.3 led to derepression and increased retrotransposition of ERVs and dysregulated a subset of endogenous adjacent genes (Elsasser, Noh et al. 2015).

Finally, H3.3 has also been implicated in regulation of imprinted genes. Failure of ATRX-dependent deposition of H3.3 to imprinted loci led to loss of H3K9me3 at these loci and hence aberrant allelic expression of these genes although the effects on expression were rather minor (Voon, Hughes et al. 2015).

1.4 Heterochromatin protein 1 (HP1)

1.4.1 Introduction to heterochromatin protein 1 (HP1)

Heterochromatin protein 1 (HP1) is a non-histone chromosomal protein that was shown in *Drosophila* by mutagenesis screen to act as a dosage dependent modifier of position effect variegation (PEV) (James and Elgin 1986, Eissenberg, James et al. 1990). HP1 homologues were subsequently observed in other eukaryotic organisms (Reviewed in - Hiragami and Festenstein 2005). Indeed, HP1 is highly conserved through evolution and its homologues are found in almost all eukaryotes except *Saccharomyces cerevisiae* in which the analogous phenomenon of telomere position effect (TPE) relies on the silent information regulatory (SIR) proteins (Singh, Miller et al. 1991).

In general, each HP1 molecule consists of two evolutionarily conserved domains: the amino terminal chromo domain (CD) and the carboxyl terminal chromoshadow domain (CSD). These two domains are joined by a hinge region (Hin) with variable length and flanked by stretches of amino acids regarded as the amino (NTE) and carboxyl (CTE) terminal extension (Figure 1.6) (Reviewed in - Hiragami and Festenstein 2005, Kwon and Workman 2011, Canzio, Larson et al. 2014). The CD of HP1 has been regarded as the chromatin binding domain with its specificity in recognising di- and tri-methylation of histone H3 lysine 9 (H3K9), a histone mark that is predominately found on silenced chromatin (Bannister, Zegerman et al. 2001, Lachner, O'Carroll et al. 2001, Jacobs and Khorasanizadeh 2002). Interestingly, delocalisation of HP1 was observed in both *S. pombe* and mouse in response to loss of an H3K9 methyltransferase (Bannister, Zegerman et al. 2001, Garcia-Cao, O'Sullivan et al. 2004). It has also been shown that removal or mutation of the CD abolishes HP1 localisation at centromeric heterochromatin (Cheutin, McNairn et al. 2003). Interestingly, different HP1 isoforms in the same organism vary in their binding affinity to H3K9 methylation. For example, the three mouse HP1 isoforms have affinity to H3K9 methylation ranging from ~5–40 μ M (Machado, Dans et al. 2010, Hiragami-Hamada, Shinmyozu et al. 2011). The CSD of HP1 is involved in mediating protein-protein interaction (Aasland and Stewart 1995) and is responsible for dimerisation of HP1 proteins forming homo- or heterodimer among or between

different HP1 isoforms (Nielsen, Oulad-Abdelghani et al. 2001). CSD of HP1 is also involved in interactions with other proteins possessing a PxVxL penta-peptide motif (Brasher, Smith et al. 2000, Cowieson, Partridge et al. 2000, Li, Kirschmann et al. 2002). However, it is noteworthy that the CSD is not the only domain mediating interaction between HP1 and other proteins as some proteins interact with HP1 through the CD or Hin regions (Reviewed in - Hiragami and Festenstein 2005). The Hin of HP1 connecting the CD and CSD is diverse in length and sequence among the HP1 isoforms. It contains a nuclear localisation sequence and sites for post-translational modifications including phosphorylation and SUMOylation (Lomberk, Bensi et al. 2006, Maison, Bailly et al. 2011) which contribute to specific functional properties of HP1 proteins. Moreover, Hin is also involved in interaction with other proteins as well as binding to DNA (Sugimoto, Yamada et al. 1996) and RNA (Muchardt, Guilleme et al. 2002). Interestingly, a recent study showed that the DNA binding ability of human HP1 α Hin is one of the determinants that contribute to the distinct recognition of the nucleosome by human HP1 α and HP1 γ (Mishima, Jayasinghe et al. 2015). Taken together, sequence diversity of Hin is the potential key for functional differentiation between different HP1 isoforms (Reviewed in - Canzio, Larson et al. 2014).

Most eukaryotes have a number of HP1 isoforms. In mammals, there are three HP1 isoforms: alpha (HP1 α), beta (HP1 β) and gamma (HP1 γ) which are encoded by the *Cbx5*, *Cbx1* and *Cbx3* genes respectively (Reviewed in - Eissenberg and Elgin 2000). HP1 isoforms show high degrees of sequence homology in their CD and CSD domains and much less in their Hin, NTEs and CTEs. For example, the CD and CSD of human HP1 α and HP1 β showed 82% sequence homology respectively while only 33% in the Hin (Figure 1.6). (Reviewed in - Canzio, Larson et al. 2014). Although having a high degree of structural and biochemical similarity (Reviewed in - Jones, Cowell et al. 2000), HP1 isoforms appeared to have distinct nuclear localisation (Horsley, Hutchings et al. 1996, Minc, Allory et al. 1999, Nielsen, Oulad-Abdelghani et al. 2001, Kourmouli, Sun et al. 2005), expression profiles (Reviewed in - Kwon and Workman 2011) and non-redundant functions (Dialynas, Terjung et al. 2007, Aucott, Bullwinkel et al. 2008, Brown, Bullwinkel et al. 2010, Lee, Li et al. 2013, Ha, Pham et al. 2014). Mammalian HP1 α and HP1 β are associated predominately with heterochromatin whereas HP1 γ was found in both

euchromatin and heterochromatin (Horsley, Hutchings et al. 1996, Minc, Allory et al. 1999, Nielsen, Ortiz et al. 1999, Minc, Courvalin et al. 2000). It has also been shown that different HP1 isoforms vary in their subnuclear localisation in different phases of the cell cycle (Minc, Allory et al. 1999). To date, factors that determine differential nuclear localisation and functional specificity or the interdependence of the HP1 protein family members remain largely unknown (Dialynas, Terjung et al. 2007).

As mentioned earlier, the CSD of HP1 can be subjected to post-translational modifications. In fact, HP1 proteins are extensively modified in a manner analogous to histones (LeRoy, Weston et al. 2009). These modifications including phosphorylation, acetylation, methylation, and formylation which have been observed in different domains of HP1 including the CD and CSD suggesting a potentially important role for these modifications in regulating HP1 activities (LeRoy, Weston et al. 2009). Some of these modifications have been implicated in specific properties and functions of HP1 proteins. For example, phosphorylated HP1 γ at serine 93 (S93P-HP1 γ) (previously regarded as serine 83) which was found to localise exclusively at euchromatic regions and serve as a marker of transcription elongation (Lomberk, Bensi et al. 2006). Recent studies have also implicated S93P-HP1 γ in gene activation (Thorne, Ouboussad et al. 2012, Harouz, Rachez et al. 2014) and alternative splicing (Saint-Andre, Batsche et al. 2011). SUMOylation of mouse HP1 α was shown to be important in targeting HP1 α to the pericentric heterochromatin (Maison, Bailly et al. 2011). Furthermore, phosphorylation of NTE of mouse HP1 α by casein kinase II (CK2) has been shown to increase HP1 α affinity to H3K9me3 peptides by approximately five-fold, bringing overall affinity of HP1 α CD and H3K9me3 similar to that of HP1 β and HP1 γ (Hiragami-Hamada, Shinmyozu et al. 2011). This modification has also been suggested to be important for proper heterochromatic localisation of HP1 α as well as maintenance of genome integrity (Hiragami-Hamada, Shinmyozu et al. 2011).

Having been shown to interact with the histone mark H3K9 methylation that associated predominately with silent heterochromatin (Bannister, Zegerman et al. 2001, Lachner, O'Carroll et al. 2001) and also to multimerise to interact with the H3K9 methyltransferase (Yamamoto and Sonoda 2003), HP1 has been regarded as

a silencing protein. However, as well as establishing and maintaining the higher-order chromatin structure (Yamamoto and Sonoda 2003), HP1 proteins are also implicated in a variety of regulatory processes including gene activation or repression, transcriptional elongation, PEV, chromosomal segregation, telomere maintenance, DNA repair and RNA processing (Reviewed in - Hiragami and Festenstein 2005, Kwon and Workman 2011, Canzio, Larson et al. 2014). In the following section, more attention will be paid to HP1 γ due to the context of this thesis.

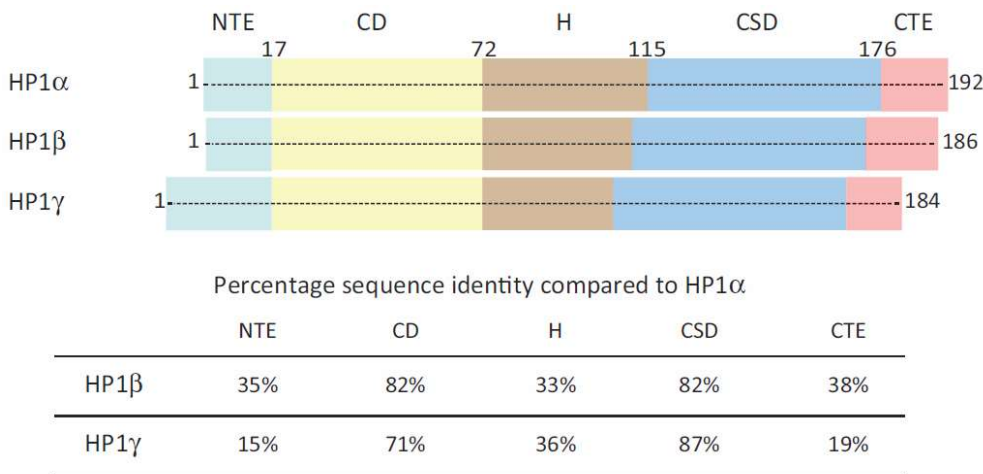


Figure 1.6 Domain maps and sequence identity of human HP1 proteins. As demonstrated by the domain maps (top) and the percentage of sequence identity (bottom) of the three human HP1 isoforms, HP1 α , HP1 β and HP1 γ showed higher degree of similarity in their chromodomain (CD) and chromoshadow domain (CSD) but vary in the hinge region (H), amino-terminal (NTE) and carboxyl-terminal (CTE) extension. This figure is reprinted from Trends in Cell Biology, Volume 24, Issue 6, Canzio, D., A. Larson and G. J. Narlikar, "Mechanisms of functional promiscuity by HP1 proteins.", 377-386, Copyright (2014), with permission from Elsevier [OR APPLICABLE SOCIETY COPYRIGHT OWNER] (Canzio, Larson et al. 2014)

1.4.2 Introduction to HP1 γ

Among the three HP1 isoforms in mammals, HP1 γ is distinguished by its unique subnuclear localisation pattern in both euchromatic and heterochromatic regions in interphase nuclei (Horsley, Hutchings et al. 1996, Minc, Allory et al. 1999, Nielsen, Ortiz et al. 1999, Minc, Courvalin et al. 2000). Indeed, HP1 isoforms were suggested to have non-redundant functions. This idea is supported not only by the difference in their subnuclear localisation patterns but also the isoform-specific binding partners identified (Reviewed in - Kwon and Workman 2008). Furthermore, by targeted deletion of each isoform, it has been shown that HP1 isoforms, except HP1 α , are essential and cannot be compensated by the other two. Mice which lack HP1 β were reported to show perinatal lethality. Moreover, newborn mice that lack HP1 β exhibited acute respiratory failure and improper brain development (Aucott, Bullwinkel et al. 2008). Although no data have been provided, the same study also reported no phenotypic difference between HP1 α wild type and deficient littermates. Of particular interest, deletion of HP1 γ is associated with neonatal lethality in mice when bred on a pure genetic background (C57BL/6) (Naruse, Fukusumi et al. 2007, Takada, Naruse et al. 2011). Although 1% of mice with a mixed genetic background (C57BL/6 and 129/Ola) were able to develop into adulthood with HP1 γ deletion (Brown, Bullwinkel et al. 2010, Takada, Naruse et al. 2011), a range of physiological defects including growth retardation and defects in germ cell development were found (Brown, Bullwinkel et al. 2010, Abe, Naruse et al. 2011, Takada, Naruse et al. 2011, Aydin, Kloos et al. 2015). Taken together, these studies indicate that HP1 γ is playing a role in the genome that cannot be compensated by the other two isoforms.

A large number proteins have been identified as binding partners of HP1 γ (Table 1.2) implicating it in a wide range of processes in both euchromatin and heterochromatin in a gene/region-specific manner. The most interesting feature of HP1 γ is probably its involvement in both repression and activation of genes. Examples implying a role for HP1 γ in gene repression include: the link to silencing of E2F- and MYC-response genes in G0 cells (Ogawa, Ishiguro et al. 2002); repression of the MMTV promoter in breast cancer cells (Vicent, Ballare et al. 2006); repression of the HIV-1 gene in conjunction with SUV39h1 (du Chene, Basyuk et

al. 2007); implicated in the SUMO E3 ligase PIAS1-mediated FOXP3 repression during T-cell differentiation (Liu, Tahk et al. 2010); implication in late pluripotency reprogramming events by repression of the pluripotency factor *Nanog* (Sridharan, Gonzales-Cope et al. 2013); as part of a repressive complex to silence 20% of hormone inducible genes in breast cancer cells (Vicent, Nacht et al. 2013) and more. Examples implying a role for HP1 γ in gene activation include: the switch from HP1 β to HP1 γ at the promoter of an inducible HIV1 gene upon activation (Mateescu, Bourachot et al. 2008) and the association of HP1 γ with the gene body of transcriptionally active genes in both human and murine cells interacting with the elongating form of RNA polymerase II (RNAPII) (Vakoc, Mandat et al. 2005, Lomberg, Bensi et al. 2006, Mateescu, Bourachot et al. 2008, Smallwood, Hon et al. 2012). In addition, S93P-HP1 γ (previously regarded as S83) was shown to serve as a marker for transcription elongation (Lomberg, Bensi et al. 2006) and gene activation (Thorne, Ouboussad et al. 2012, Harouz, Rachez et al. 2014). Of particular interest, HP1 γ has been shown to interact with the histone variant H3.3 which associates predominately with actively transcribed genes. Intriguingly, the binding of both H3.3 and HP1 γ to the HSP70 genes was found to be interdependent and correlated with the level of HSP70 gene expression (Kim, Heo et al. 2011). The exact nature and mechanisms of how the active or repressive role of HP1 γ is determined remains unclear. One might expect that the mode of HP1 γ regulation is dependent on the ‘context’ i.e. which regulatory factors (including specific epigenetic modifications) it interacts with in different nuclear environments (e.g. euchromatic or heterochromatic). This thesis will address this hypothesis experimentally by focusing on the functional interdependence between H3.3 and HP1 γ *in vivo* (see section 1.10 for aims of this project).

Apart from regulation of gene expression, HP1 γ has also been implicated in the regulation of processes including RNA splicing (Saint-Andre, Batsche et al. 2011, Smallwood, Hon et al. 2012, Salton, Voss et al. 2014) and transcriptional termination (Skourti-Stathaki, Kamieniarz-Gdula et al. 2014). In heterochromatic regions, HP1 γ has been shown to be involved in maintenance of sister-telomere cohesion and telomere length (Canudas, Houghtaling et al. 2011). Moreover, knockdown of HP1 γ was shown to prevent p53-mediated alternative lengthening of telomeres (ALT)-associated PML nuclear bodies (APB) formation which is found

exclusively in telomerase-negative cancer cells which use ALT as the main mechanism to maintain telomeres. Also, loss of HP1 γ was reported to be associated with mitotic aberrations in a number of studies (Obuse, Iwasaki et al. 2004, Serrano, Rodriguez-Corsino et al. 2009, Shimura, Toyoda et al. 2011, Grzenda, Leonard et al. 2013). More recently, HP1 γ has also been implicated in DNA damage response in a number of studies (Lee, Kuo et al. 2013, Soria and Almouzni 2013, Akaike, Kuwano et al. 2014, Wu, Nishikawa et al. 2015).

Protein	Species	Interacting domain	Reference(s)
<u>Chromatin associated factors</u>			
H1 (K29me2)	Human	CD	1
H3	Mouse	CD	2
H3.3	Human	nd	3, 4
H3K9me3	Human, mouse	CD	5, 6
HP1α	Human, mouse	CSD	2, 7
HP1β	Human, mouse	CSD	2, 7
HP1γ	Human, mouse	CSD	2, 7
INCENP	Human	nd	8
TIN2	Human	CSD	9
<u>Chromatin modifiers</u>			
DNMT1	Human	CD	10
DNMT3a	Human	nd	10
DNMT3b	Human	nd	10
G9a	Human	nd	11
G9a (K185me3)	Human	CD	1, 12
KDM2A	Human	nd	13
PIM1	Human	CSD	14
SUV39H1	Human	nd	11, 15
SUV4-20H2	Mouse	nd	16
<u>Transcription associated factors</u>			
BRCA1	Human	nd	17
E2F-6	Human	nd	12
IKKα	Mouse	nd	18
MED29	Mouse	nd	19
PAX3	Human	nd	20
RNA	Mouse	nd	21
RNAPII	Human, mouse	nd	18, 22-25
TAFII130 (of TFIID)	Human	CSD	26
TIF1α	Mouse	CSD	27
TIF1β	Human, mouse	CSD	27, 28
<u>Splicing associated factors</u>			
AGO2	Human	nd	29
SRSF1	Human	nd	30
<u>Proteins involved in DNA repair</u>			
BARD1	Human	nd	31
HIPK2	Human	nd	32
KU70	Human	CSD	24
<u>Proteins involved in nuclear organisation</u>			
hMIS12 (kinetochore protein)	Human	nd	33
LAMIN B receptor (LBR)	Human	CSD	34, 35
Nuclear envelope	Mouse	CD	36

Table 1.2 Examples of mammalian HP1 γ interacting partners. CD, chromodomain; CSD, chromoshadow domain; nd, not determined. References used are:

(Ruan, Ouyang et al. 2012)¹, (Nielsen, Oulad-Abdelghani et al. 2001)², (Kim, Heo et al. 2011)³, (Loyola, Bonaldi et al. 2006)⁴, (Lachner, O'Carroll et al. 2001)⁵, (Bannister, Zegerman et al. 2001)⁶, (Rosnoblet, Vandamme et al. 2011)⁷, (Ainsztein, Kandels-Lewis et al. 1998)⁸, (Canudas, Houghtaling et al. 2011)⁹, (Smallwood, Esteve et al. 2007)¹⁰, (Fritsch, Robin et al. 2010)¹¹, (Ogawa, Ishiguro et al. 2002)¹², (Frescas, Guardavaccaro et al. 2008)¹³, (Koike, Maita et al. 2000)¹⁴, (Stewart, Li et al. 2005)¹⁵, (Schotta, Lachner et al. 2004)¹⁶, (Choi, Park et al. 2012)¹⁷, (Thorne, Ouboussad et al. 2012)¹⁸, (Sridharan, Gonzales-Cope et al. 2013)¹⁹, (Hsieh, Yao et al. 2006)²⁰, (Muchardt, Guilleme et al. 2002)²¹, (Mateescu, Bourachot et al. 2008)²², (Smallwood, Hon et al. 2012)²³, (Lomberg, Bensi et al. 2006)²⁴, (Vakoc, Mandat et al. 2005)²⁵, (Vassallo and Tanese 2002)²⁶, (Lechner, Begg et al. 2000)²⁷, (Brasher, Smith et al. 2000)²⁸, (Ameyar-Zazoua, Rachez et al. 2012)²⁹, (Salton, Voss et al. 2014)³⁰, (Wu, Nishikawa et al. 2015)³¹, (Akaike, Kuwano et al. 2014)³², (Obuse, Iwasaki et al. 2004)³³, (Polioudaki, Kourmouli et al. 2001)³⁴, (Ye, Callebaut et al. 1997)³⁵, (Kourmouli, Theodoropoulos et al. 2000)³⁶

1.5 Genomic imprinting

Genomic imprinting is an epigenetic phenomenon that results in mono-allelic expression of the identical gene according to the parental inheritance. To date, more than 100 genes in mouse were identified as imprinted genes (MouseBook™). A lot of these imprinted genes are implicated in growth and development and also other processes including energy homeostasis, brain function and behaviour (Reviewed in - Nordin, Bergman et al. 2014). Most of the imprinted genes are found in small clusters (Reviewed in - Regha, Sloane et al. 2007) and the allelic repression is controlled by imprinted control regions (ICRs) (Reviewed in - Delaval and Feil 2004). ICRs of either the maternally or paternally inherited allele are marked by DNA methylation which provides parental allele specific function to the ICR and hence mediates imprinted gene expression (Reviewed in - Kelsey and Feil 2013). In general, the imprinted (silent) allele is associated with DNA methylation and inactive histone modifications while the active allele is associated with active histone marks (Pedone, Pikaart et al. 1999, Singh, Wu et al. 2011).

A classic example of an imprinted gene cluster is the insulin-like growth factor 2 (*Igf2*)/*H19* cluster. *Igf2* and *H19* genes are reciprocally expressed from two parental alleles. *Igf2* is expressed only from the paternal allele and encodes the Igf2 protein which is responsible for promoting both foetal and placental growth (Constancia, Hemberger et al. 2002). In mice, the *Igf2* gene is silent in almost all tissues after birth (Constancia, Hemberger et al. 2002). *H19* is transcribed only from the maternal allele but not translated to a protein (Reviewed in - Nordin, Bergman et al. 2014). Rather, *H19* encodes a 2.3 kb non-coding mRNA (ncRNA) which is abundantly expressed in both embryonic and extraembryonic tissues during development. However, the exact role of *H19* ncRNA is still unclear (Reviewed in - Nordin, Bergman et al. 2014).

A well-accepted model for regulation of the *Igf2*/*H19* imprinting cluster involves ICR1 which is a genomic region located approximately 2-4 kb upstream of the *H19* transcription start site (Figure 1.7) (Thorvaldsen, Duran et al. 1998). ICR1 is rich in CpG residues which allow differential methylation state of the two alleles. The paternal allele is methylated whereas the maternal allele is unmethylated and allows

binding of the insulator protein CTCF. Binding of CTCF to ICR1 on the maternal allele prevents the common enhancer for both *Igf2* and *H19* gene from acting on the *Igf2* gene leading to its repression (Thorvaldsen, Duran et al. 1998, Thorvaldsen, Fedoriw et al. 2006). Methylation of ICR1 on the paternal allele prevents CTCF binding and allows paternal *Igf2* expression (Bell and Felsenfeld 2000, Hark, Schoenherr et al. 2000). Little is known about potential global regulation of imprinted genes although it is possible that HP1 is involved. This will be experimentally addressed in this thesis (see result section 3.1.7).

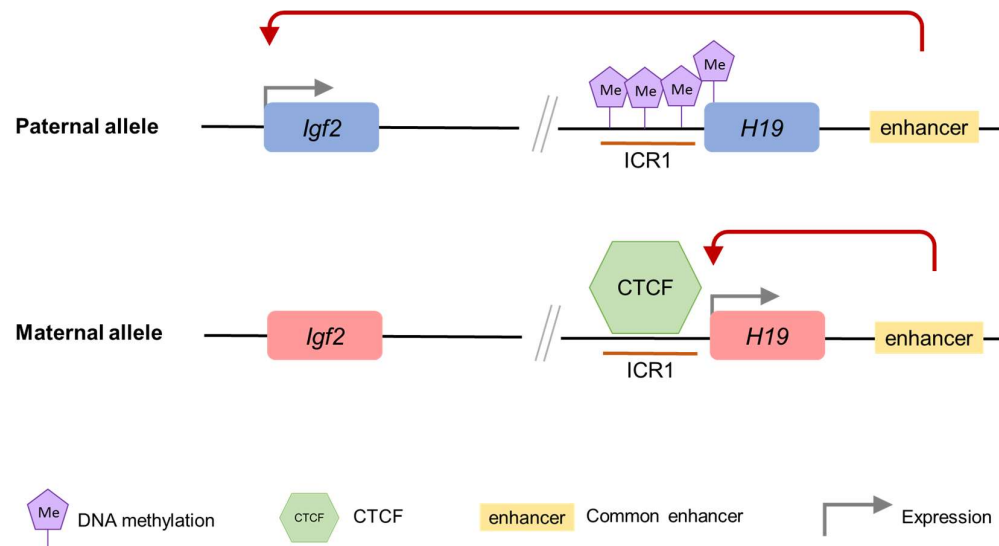


Figure 1.7 Schematic diagram demonstrating model for regulation of *Igf2/H19* imprinting cluster by ICR1. ICR1 which locates upstream of the *H19* transcription start site is rich in CpG residues which allow differential methylation state of the two alleles. Methylated paternal allele inhibits binding of CTCF. The common enhancer can then act on the *Igf2* and allow its paternal expression. Binding of CTCF to ICR1 on the unmethylated maternal allele prevents the common enhancer from acting on the *Igf2* gene. Maternal *H19* is hence expressed.

1.6 Position Effect Variegation (PEV)

When some genes are abnormally juxtaposed by rearrangement or transposition from their original euchromatic position to a location close to heterochromatin, they show variegation of expression resulting in stochastic silencing of the affected gene in a proportion of cells that would normally express it. This phenomenon, resulting from changing the position of the gene in the genome is termed 'position effect variegation' (PEV). These rearrangements are thought to remove the normally existing genetic boundary elements or 'buffer zone', allowing 'spreading' of heterochromatin along the chromosome from the adjacent constitutive heterochromatin and hence lead to silencing (Reviewed in - Elgin and Reuter 2013). However, whether this is regulated by multiple processes or a dominant mechanism remain unclear (Reviewed in - Brummelkamp and van Steensel 2015). It is thought that the decision to activate or silence in PEV occurs early in the development of a specific lineage and this decision is maintained by epigenetic modifications as cells progress through successive cell divisions (Reviewed in - Festenstein and Kioussis 2000).

PEV was first described by Hermann Muller in 1930 with the observation that translocation of the *Drosophila white* gene in close proximity to a block of centromeric heterochromatin resulted in variegation of red eye colour in a proportion of eye cells in *Drosophila* (Muller and Altenburg 1930) (Figure 1.8). Phenomena that resemble PEV have subsequently been observed in other organisms including yeasts (Allshire, Javerzat et al. 1994) and mammals (Cattanach 1974, Pravtcheva, Wise et al. 1994, Festenstein, Tolaini et al. 1996, Milot, Strouboulis et al. 1996). One of the examples of a phenomenon similar to PEV that occurs in mammals includes the variable silencing of a coat colour gene in mice upon insertion onto the inactive X chromosome in females as a consequence of X inactivation by heterochromatinisation (Cattanach 1961, Russell and Bangham 1961). It has also been shown that silencing of CD2 and β -globin transgenes was induced when integrated into the pericentromeric regions (Festenstein, Tolaini et al. 1996). Intriguingly, PEV-induced silencing of these transgenes can be overcome completely by the addition of locus control regions (LCRs) on the transgene (Festenstein, Tolaini et al. 1996, Milot, Strouboulis et al. 1996). These are gene

regulatory elements that control the establishment of gene expression in *cis* in all cells of a given cell lineage (Reviewed in - Kioussis and Festenstein 1997). Of particular interest, the variegation phenotype was also observed when the CD2 transgene was put in close proximity to DNA triplet repeats found in human diseases including the GAA repeats associated with Friedreich's Ataxia (Saveliev, Everett et al. 2003). This observation led to the hypothesis that the silencing effect induced by pathological triplet repeat expansion might be exerted by mechanisms resembling heterochromatin-mediated PEV (Saveliev, Everett et al. 2003).

A large number of PEV modifier which suppress (suppressor of variegation (Su(var))) or enhance (enhancer of variegation (E(var))) PEV were identified by mutagenesis screens of *Drosophila* (Reviewed in - Wallrath 1998). These PEV modifiers display dosage dependent effects whereby overexpression of Su(var)s enhances heterochromatic gene silencing and the downregulation causes suppression of PEV or vice versa for E(var)s (Reviewed in - Festenstein and Kioussis 2000). Of note, two of the suppressors of variegation identified include heterochromatin protein 1 (HP1) and the H3K9 methyltransferase Su(var)3-9 that are homologues of mammalian HP1 and SUV39H respectively (Reviewed in - Elgin and Reuter 2013).

Drosophila X chromosome

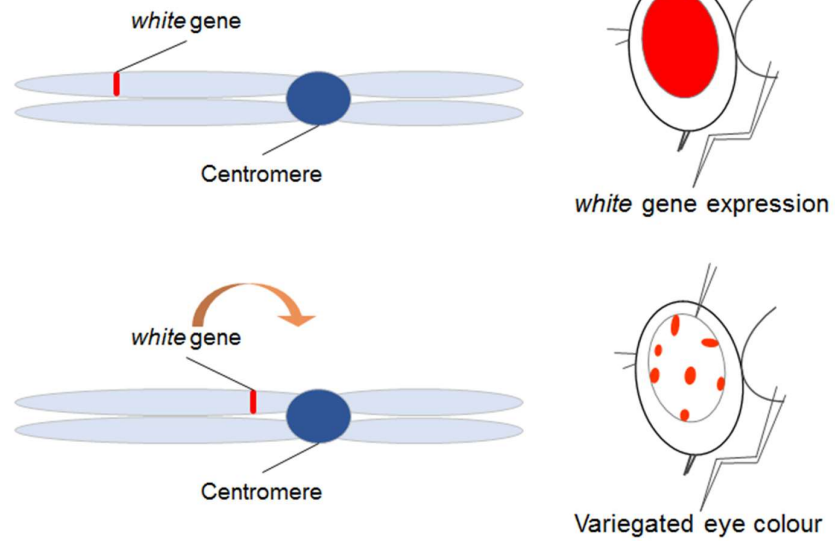


Figure 1.8 Model of position effect variegation (PEV) in *Drosophila*. Translocation of the *white* gene to the proximity of centromere caused the variegation of *white* gene expression and hence the variegated phenotype (red eye colour) in a proportion of cells in the *Drosophila* eye.

1.7 Sexual dimorphism

Sex dimorphism refers to the genotypic and phenotypic differences between males and females of the same species. The fact that specific diseases may be more prevalent and severe in one gender than the other (Miller, Kaplan et al. 2011, Voskuhl 2011, Karastergiou, Smith et al. 2012, Sandberg and Ji 2012) increases our appreciation and interest in sex differences in physiology and disease (Reviewed in - Arnold 2014).

Sex differences are determined by three main factors: activational effect of gonadal steroid hormones, organisational effects of gonadal steroid hormones and sex chromosomes (Reviewed in - Arnold 2009). Hormonal secretion by testes and ovaries in adult (predominately of androgens, estrogens and progestins) act on different tissues to induce gonadal phenotypes in males and females (Reviewed in - Rinn and Snyder 2005, Isensee and Ruiz Noppinger 2007). These hormonal effects are reversible as they are eliminated by adult gonadectomy in hours to weeks and classified as activational effects (Reviewed in - Arnold 2014). However, some sexual differences driven by gonadal hormones cannot be eliminated after gonadectomy. Examples include the sexual differentiation of the genitals as well as the specific sexual differences in the brain and behaviour (Jost, Vigier et al. 1973, Arnold and Gorski 1984, Breedlove, Cooke et al. 1999, McCarthy and Arnold 2011). These long-lasting or permanent changes caused by gonadal hormones are referred to as organisational effects (Phoenix, Goy et al. 1959).

The primary sex-determining factors are encoded by the sex chromosomes (X and Y chromosomes) which are the only factors that differ in male and female zygotes and thus give rise to all downstream sex differences (Reviewed in - Arnold, Chen et al. 2012). All sexual differences are hence under the control of a genetic imbalance conferred by the gender-specific sex chromosome complements ('XX' in females and 'XY' in males) (Burgoyne 1993, Thornhill and Burgoyne 1993, Burgoyne, Thornhill et al. 1995).

The male specific Y chromosome harbors male-specific genes that act only in XY cells. It carries the testis-determining *Sry* gene (Reviewed in - Goodfellow and

Lovell-Badge 1993) as well as other genes that are critical for spermatogenesis (Reviewed in - Arnold 2014). The X chromosomes can be found in both males and females. While one of the X chromosomes in females is inactivated by epigenetic mechanisms, X genes escaping X inactivation may then be expressed at a higher level in XX than XY cells (Reviewed in - Berletch, Yang et al. 2010, Arnold 2014). Moreover, as females inherit X chromosomes from both parents, female XX cells receive both paternal and maternal imprints on X chromosomes while male XY cells receive only maternal imprint. The effects of imprinting on X chromosomes in female cells thus depends on which X chromosome is active in a specific cell. This difference in male and female cells could hence contribute to sexual dimorphism (Reviewed in - Arnold, Chen et al. 2012). Furthermore, the sex-specific heterochromatic state of X and Y chromosome segments may alter the availability of factors which modulate epigenetic status of all chromosomes and hence regulate gene expression on autosomes in a sex-specific manner. The existence of this sex specific effect is supported by the studies in *Drosophila* showing that the large heterochromatic Y chromosome may act as a ‘sink’ for heterochromatic factors (indeed it is a powerful modifier of PEV) and thereby alter gene expression of autosomal genes by affecting the epigenetic status of the autosomes due to a relative deficiency in heterochromatic factors (Jiang, Hartl et al. 2010, Lemos, Branco et al. 2010). Although mechanistic evidence for a similar Y chromosome effect is lacking in mammalian cells where the Y chromosome is relatively small (Reviewed in - Arnold 2014), the heterochromatic inactive X chromosome present only in XX mammalian cells could alter the availability of heterochromatic factors and hence the epigenetic status of specific autosomal loci (Wijchers, Yandim et al. 2010, Wijchers and Festenstein 2011). Indeed, the sex chromosome complement sensitive genes were enriched for genes that are repressed by HP1 β (Wijchers, Yandim et al. 2010) which suggested that HP1 might be a regulator of sexually dimorphic gene expression – a hypothesis which has been directly addressed in this thesis (see section 1.10 for aims of this project).

1.8 Friedreich's Ataxia (FRDA)

Friedreich's Ataxia (FRDA) is a DNA repeat-expansion disease where the repeats have been implicated in aberrant gene silencing at the heterochromatin level. It is an autosomal recessive neurodegenerative disease (Campuzano, Montermini et al. 1996). It is the most common inherited ataxia in Caucasians affecting 1 in 30,000 to 50,000 people (Reviewed in - Koeppen 2011, Koeppen 2013). This disease normally presents in childhood (Reviewed in - Festenstein 2006) primarily affecting the nervous system and the heart (Campuzano, Montermini et al. 1996). FRDA features a range of symptoms and signs including dis-coordination, muscle weakness, dysarthria, diabetes mellitus, peripheral neuropathy, cardiomyopathy and frequently leads to severe disability by early adulthood (Reviewed in - Festenstein 2006, Schmucker and Puccio 2010). At present, no effective disease modifying treatment is available for FRDA (Reviewed in - Libri, Yandim et al. 2014).

FRDA is caused by the deficiency of the mitochondrial protein frataxin which is evolutionarily conserved and expressed in all cell types (Reviewed in - Schmucker and Puccio 2010). Deficiency of frataxin leads to severe disruption of iron-sulfur cluster biosynthesis, overload of mitochondrial iron and increased sensitivity of the cells to oxidative stress which subsequently results in cell death (Reviewed in - Schmucker and Puccio 2010). It has been shown that silencing of the *FXN* gene by RNA interference causes large scale cell death in differentiated neuron-like cells associated with upregulation of pro-apoptotic factors including p53, PUMA and Bax (Palomo, Cerrato et al. 2011).

The majority of cases of FRDA are caused by pathological expansion of a trinucleotide GAA repeat within intron 1 of the *FXN* gene, leading to partial silencing of the gene and hence deficiency of frataxin protein (Campuzano, Montermini et al. 1996). Healthy individuals normally have 8-33 GAA repeats while FRDA patients carry ~70 to more than 1000 triplet repeats (Durr, Cossee et al. 1996). Most FRDA patients carry GAA repeat expansions at both alleles which lead to a large reduction in *FXN* expression in all tissues to only 10-25% of normal levels (Campuzano, Montermini et al. 1996, Campuzano, Montermini et al. 1997). A small fraction of FRDA patients are compound heterozygous who have GAA

repeat expansions at only one *FXN* allele and a point mutation on the other allele (Cossee, Durr et al. 1999, Gellera, Castellotti et al. 2007). Due to the recessive nature of the disease, heterozygous carriers have only one affected allele and are asymptomatic and have ~50% of normal frataxin levels (Campuzano, Montermini et al. 1997, Pandolfo 2002).

The molecular mechanisms by which GAA repeat expansions induce *FXN* gene silencing have been studied intensively and two non-exclusive models have emerged. Firstly, it has been shown by numerous biochemical studies that GAA repeat expansions can adopt abnormal DNA structures such as non-B DNA conformation (Ohshima, Montermini et al. 1998), the so-called ‘sticky DNA’ structure (Sakamoto, Chastain et al. 1999, Sakamoto, Ohshima et al. 2001) as well as RNA-DNA hybrids (Grabczyk, Mancuso et al. 2007). These structures can potentially act as a physical blockage for the elongating RNAPII to pass through the *FXN* gene (Sakamoto, Ohshima et al. 2001). Secondly, it has been suggested that GAA repeat expansions may silence the *FXN* gene by inducing heterochromatin formation through epigenetic mechanisms (Reviewed in - Schmucker and Puccio 2010, Yandim, Natisvili et al. 2013). This idea originated following the discovery that expanded GAA repeats can induce silencing of a linked heterochromatin-sensitive human reporter gene (hCD2) in transgenic mice resulting in a phenomenon that resembled PEV (Saveliev, Everett et al. 2003). Moreover, this study has also shown that GAA repeats-induced variegated silencing could be enhanced by overexpression of the PEV modifier HP1 β (Saveliev, Everett et al. 2003). Heterochromatin-mediated silencing triggered by GAA repeat expansions was further supported by several more recent studies showing that the chromatin of the flanking regions of *FXN* alleles with GAA repeat expansions have hypoacetylation of histone H3 and H4 as well as methylation of histone H3K9 and H3K27, modifications that are characteristic of transcriptionally silent heterochromatin (Herman, Jenssen et al. 2006, Greene, Mahishi et al. 2007, Al-Mahdawi, Pinto et al. 2008, Rai, Soragni et al. 2008, Chan, Torres et al. 2013, Libri, Yandim et al. 2014). Intriguingly, two recent clinical studies have shown that oral administration of histone deacetylase (HDAC) inhibitors by patients is able to increase frataxin level with associated epigenetic changes at the *FXN* locus. Libri *et al* showed that oral intake of the HDAC inhibitor nicotinamide is able to increase

both FXN mRNA and frataxin protein level towards asymptomatic carriers' level and reduce the level of trimethylated H3K9 on the *FXN* gene in primary lymphocytes derived from FRDA patients (Libri, Yandim et al. 2014). Soragni *et al* showed that FRDA patients treated with a novel class II HDAC inhibitor have increased FXN mRNA level as well as H3K9 acetylation in their lymphocytes (Soragni, Miao et al. 2014). Such results suggest that HP1 might be responsible for the aberrant silencing in FRDA (see section 3.4).

1.9 Genome-wide studies of the transcriptome and epigenome

1.9.1 Overview

The human genome is comprised of approximately 25,000 genes which give rise to >80,000 protein coding transcripts and estimated to produce 250,000 to 1 million proteins (Reviewed in - de Klerk and 't Hoen 2015). The large number of transcripts and proteins per gene indicate the complexity of gene regulation mechanisms/networks which take place at different levels: genetic, epigenetic, transcriptional, post-transcriptional and translational level. In the last decade, methodologies which are based on next-generation sequencing for genome-wide profiling of the transcriptome (Nagalakshmi, Wang et al. 2008), the epigenome (Barski, Cuddapah et al. 2007, Johnson, Mortazavi et al. 2007, Robertson, Hirst et al. 2007) and chromosome conformation (Reviewed in - de Wit and de Laat 2012) have emerged. The transcriptome is defined as the complete set of RNA transcripts and their quantity in a cell (Wang, Gerstein et al. 2009) while the epigenome is defined as the landscape of epigenetic regulatory factors across the genome (Bernstein, Meissner et al. 2007). These technologies provide global insight and enhance our understanding of the complex genome. They also allow extensive comparison between cells at different developmental stages or under different physiological conditions. In this section, the features of the methodologies looking at the transcriptome and epigenome will be introduced.

1.9.2 RNA sequencing (RNA-seq) for studying the transcriptome

RNA sequencing (RNA-seq) is a methodology which provides profiling and quantification of the transcriptome based on next generation sequencing and allows measurement and comparison of gene expression patterns at high resolution (Reviewed in - Finotello and Di Camillo 2015). RNA-seq is not limited to measuring messenger RNA (mRNA) transcripts derived from protein coding genes but also small RNAs and non-coding RNAs (Reviewed in - Wang, Gerstein et al. 2009). High accuracy of RNA-seq for quantifying expression levels has been confirmed using qRT-PCR (Nagalakshmi, Wang et al. 2008) and 'spike-in' RNA

controls of known concentration (Mortazavi, Williams et al. 2008). As RNA-seq is a sequence-based approach which directly determines the cDNA sequences, it can be used to detect transcripts that do not correspond to existing genomic sequence (Reviewed in - Wang, Gerstein et al. 2009). This feature makes RNA-seq an attractive tool for non-model organisms with undetermined genomic sequences (Vera, Wheat et al. 2008). Moreover, the high resolution of RNA-seq to a single base allows the precise localisation of transcription boundaries (Reviewed in - Wang, Gerstein et al. 2009) as well as revealing sequence variations such as single nucleotide polymorphisms (SNPs) in the transcribed regions (Cloonan, Forrest et al. 2008, Morin, Bainbridge et al. 2008). In conjunction with novel computational tools, RNA-seq is also a powerful tool for determining the expression of alternative RNA isoforms produced by the key regulatory process of alternative splicing (Anders, Reyes et al. 2012).

1.9.3 Chromatin immunoprecipitation sequencing (ChIP-seq) for studying the epigenome

Chromatin immunoprecipitation (ChIP) was developed more than one decade ago for mapping epigenetic features including histone modifications and transcription factors' binding sites across different genomic regions in their native chromatin context (Ren, Robert et al. 2000, Iyer, Horak et al. 2001, Lieb, Liu et al. 2001, Horak and Snyder 2002, Weinmann, Yan et al. 2002). Hence, *in vivo* localisation of these epigenetic factors can be determined. The sequence identities of the precipitated DNA fragments in ChIP experiments are commonly detected by standard PCR and qRT-PCR although Southern and western blot analysis have also been used (Orlando, Strutt et al. 1997, Singal, vanWert et al. 2002, Wells and Farnham 2002). However, these methods are associated with the limitation that existing knowledge of the target gene is required in order to design specific primers and probes for detection. More recently, ChIP followed by high-throughput sequencing (ChIP-seq) has been developed (Barski, Cuddapah et al. 2007, Johnson, Mortazavi et al. 2007, Robertson, Hirst et al. 2007). This technique allows identification of regions of a genome that are associated with a given protein or epigenetic modification and assessment of their relative enrichment in a genome-wide manner, hence enabling characterisation

of the ‘epigenome’. The data obtained from ChIP-seq can be at high resolution as it provides actual DNA sequences of the precipitated fragments (Reviewed in - Mundade, Ozer et al. 2014).

In order to obtain high quality ChIP-seq data, several technical aspects have to be properly controlled. Firstly, being a technique which relies on the use of antibody, the quality of antibody used is key. Antibodies should therefore provide high specificity and sensitivity to allow detection of enrichment peaks without substantial background noise (Reviewed in - Kidder, Hu et al. 2011). In cases where antibodies of good quality are not available, epitope-tagged proteins can be employed. In this strategy, epitope-tagged proteins are expressed in targeted model systems and antibody recognising the epitope can be employed for ChIP. Examples of commonly used epitopes include hemagglutinin (HA), Flag, Myc, V5 and green fluorescent protein (GFP) (Kolodziej, Pourfarzad et al. 2009, Goldberg, Banaszynski et al. 2010, Chen, Stempor et al. 2014). Secondly, sufficient numbers of cells per ChIP reaction are required to help ensure a high signal-to-noise ratio. In order to control for bias in chromatin fragmentation and sequencing reactions, ‘input’ control which reflects the total material used for ChIP is necessary. Specificity of antibody used can be controlled by performing ChIP on samples with the factor of interest depleted. In this case, as the level of target protein is diminished to background level, any binding events revealed can be assumed to be non-specific. Moreover, biological replicates of experiments are required to ensure reliability of data. Although there is no consensus on the best number of replicates for ChIP-seq, a minimum of duplicate biological experiments has been suggested (Chen, Negre et al. 2012). Furthermore, DNA fragments of about 150-300 bp which is equivalent to mono- and dinucleosome chromatin fragments have been considered to be the optimal size-range of chromatin for ChIP-seq analysis. DNA fragments of this size provide good resolution for ChIP-seq analysis and work well for next-generation sequencing platform (Reviewed in - Chen, Negre et al. 2012). Finally, the number of sequencing reads has to be sufficient for reasonable genomic coverage. The number of sequencing reads required is dependent on the affinity of antibody as well as the abundance of target sites in the genome (Reviewed in - Chen, Negre et al. 2012).

1.10 Aims of this project

Heterochromatin protein 1 (HP1) is a non-histone chromosomal protein that was shown in *Drosophila* by mutagenesis screen to act as a dosage dependent modifier of the archetypal epigenetic phenomenon of position effect variegation (PEV). HP1 γ , one of the mammalian HP1 isoforms is distinguished by its subnuclear localisation to both heterochromatic and euchromatic regions within the nucleus. Interestingly, it is implicated in a wide range of processes in both euchromatin and heterochromatin in a gene/region-specific manner and involved in both repression and activation of genes. It has been shown to bind to the histone modification H3K9me3 (Bannister, Zegerman et al. 2001, Lachner, O'Carroll et al. 2001) and interact with the histone variant H3.3 (Loyola, Bonaldi et al. 2006, Kim, Heo et al. 2011). In the triplet-repeat expansion disease, Friedreich's Ataxia, the *FXN* gene is heterochromatinised and silenced and decorated with H3K9me3 and HP1 γ binding (De Biase, Chutake et al. 2009, Chan, Torres et al. 2013). However, the exact nature and mechanisms of how the active or repressive role of HP1 γ is determined remains uncertain. This project set out to investigate the role of HP1 γ by a classical gene knockout and knockdown approach.

In this project, using mouse embryonic fibroblasts which are wild type or homozygous knockout for HP1 γ . I aimed to experimentally address the following general questions:

- What is the role of HP1 γ in the regulation of gene expression?
- What is the role of HP1 γ in the regulation of heterochromatin?
- Is the activatory or repressive role of HP1 γ correlated with its association with the interacting histone variant H3.3?
- Can the aberrantly silenced *FXN* gene be re-activated by decreasing HP1 γ dosage?
- Does HP1 γ participate in regulation of sexually dimorphic gene expression?
- Does HP1 γ regulate imprinted genes?

Chapter 2 - Materials and Methods

2.1 Generation of Mouse Embryonic fibroblasts (MEFs)

Pregnant female mice were sacrificed at day 13.5 of gestation (E13.5) (considering the day of vaginal plug found as day 0). The uterus was obtained and placed in cold 1x PBS. Embryos were removed from the uterus and transferred to a fresh petri dish containing cold 1x PBS. Under the light microscope, placenta and yolk sack were separated from the embryo and the head and internal organs were removed. The body of embryo was transferred to 1ml of PBS and finely minced. 1.5 ml of 0.25% trypsin-EDTA (Sigma) was added to the suspended tissue and incubated at 37°C for 1.5min. To inactivate trypsin, 10ml of culture medium was added to the suspension and the sample was passed through a 0.7µm cell strainer. The cell strainer was rinsed once with 10ml culture medium giving a final cell suspension volume of 22.5ml. Cells in the suspension were then collected by centrifugation at 300 x g at room temperature for 5min. The supernatant was discarded and the cell pellet was resuspended in 5ml culture medium, transferred to a 6cm culture dish and incubated overnight at 37°C, 5% CO₂. Culture medium was replaced the following day. The cells were then cultured and expanded according to growth rate. Culturing conditions and details of genotyping can be found in later sections (2.2.2.1 and 2.4).

2.2 Cell culture

2.2.1 Culturing and Passaging

2.2.1.1 Mouse Embryonic fibroblasts (MEFs)

MEF cells were cultured until 95% confluent in 10cm dishes. Culture media were discarded and the cells were washed once with 1x PBS. Cells were detached by adding 1ml of 0.25% Trypsin-EDTA per dish followed by 3 min incubation at 37°C for 5 min. Trypsin was inactivated by addition of 5ml culture media and the cells were transferred to Falcon tubes. The cells were collected by centrifugation at 300 x g at room temperature for 5 min. Supernatant was discarded and the cell pellet was resuspended in fresh culture media. One-third of cells were seeded into each fresh 10cm dish with final culture media volume as 10ml and cultured at 37°C, 5% CO₂.

2.2.1.2 Human fibroblast cell lines

Human fibroblast cell lines derived from healthy individuals or FRDA patients used in this project were purchased from the Coriell Cell Repository, Camden, New Jersey, USA. Human fibroblast cell lines were cultured until 95% confluent in 10cm dishes. Culture media were discarded and the cells were washed once with 1x PBS. Cells were detached by adding 1ml of 0.25% Trypsin-EDTA per dish followed by 5 min incubation at 37°C for 5 min. Trypsin was inactivated by addition of 5ml culture media and the cells were transferred to falcon tube. The cells were collected by centrifugation at 300 x g at room temperature for 5 min. Supernatant was discarded and the cell pellet was resuspended in fresh culture media. One-fourth of cells were seeded into each fresh 10cm dish with a final culture media volume of 10ml and cultured at 37°C, 5% CO₂.

Human fibroblast cell lines used:

Coriell Catalog number	Cell type	Species	Pheno-type	GAA-repeat size	Gender	Race	Age of sample taken (Years)
GM07492	Fibroblast	Homo Sapiens	Un-affected	6-34 repeats (normal)	Male	Caucasian	17
GM04503	Fibroblast	Homo Sapiens	Un-affected	6-34 repeats (normal)	Female	Caucasian	31
GM04078	Fibroblast	Homo Sapiens	FRDA	541/420 in either alleles	Male	Caucasian	30
GM03665	Fibroblast	Homo Sapiens	FRDA	445/740 in either alleles	Female	Caucasian	13

2.2.1.3 Human Embryonic fibroblast 293T cells (HEK293T)

HEK293T cells were cultured until 95% confluent in 10cm dishes. Culture medium was discarded and the cells were washed once with 1x PBS. Cells were detached by adding 1ml of 0.25% Trypsin-EDTA per dish followed by 2 min incubation at 37°C for 5min. Trypsin was inactivated by addition of 5ml culture media and the cells were transferred to falcon tubes. The cells were collected by centrifugation at 300 x g at room temperature for 5 min. Supernatant was discarded and the cell pellet was resuspended in fresh culture media. One-fourth of cells were seeded into each fresh 10cm dishes with a final culture media volume of 10ml and cultured at 37°C, 5% CO₂.

2.2.1.4 HeLa cells

HeLa cells were cultured until 95% confluent in 10cm dishes. Culture medium was discarded and the cells were washed once with 1x PBS. Cells were detached by adding 1ml of 0.25% Trypsin-EDTA per dish followed by 2 min incubation at 37°C for 5 min. Trypsin was inactivated by addition of 5ml culture media and the cells were transferred to falcon tubes. The cells were collected by centrifugation at

300 x g at room temperature for 5 min. Supernatant was discarded and the cell pellet was resuspended in fresh culture media. One-fifth of cells were seeded into each fresh 10cm dishes with final culture media volume of 10ml and cultured at 37°C, 5% CO₂.

2.2.2 Freezing and thawing cells

10⁶ viable human fibroblasts and HeLa cells or 95% confluent dish of MEFs (passage 1) and HEK293T cells were harvested. The cell pellets were resuspended in freezing solution (90% FBS, 10%DMSO) and transferred to 2ml Cryovial. The cells were then frozen down in a Nalgene® Cryo 1°C “Mr. Frosty” freezing container and kept at -80 °C overnight. Cryovials were stored in liquid nitrogen the next day.

To recover the cells, the cryovial was taken out from liquid nitrogen and place immediately in a 37°C water bath. The cell mixture was then transferred into 5 ml culture medium and centrifuged at 300 x g at room temperature for 5 min. Supernatant was discarded and the cell pellet was resuspended in 10ml of fresh culture medium, transferred to 10ml dish and culture at 37°C, 5% CO₂.

2.2.3 Culture media

MEFs and human fibroblast cell lines:

DMEM high glucose (GIBCO ®), Fetal bovine serum (Sigma) (15% v/v), Penicillin (GIBCO ®) (1% v/v), GlutaMAX™-I 100x (GIBCO ®) (1% v/v)

HEK293T and HeLa cells:

DMEM high glucose (GIBCO ®), Fetal bovine serum (Sigma) (10% v/v), Penicillin (GIBCO ®) (1% v/v), GlutaMAX™-I 100x (GIBCO ®) (1% v/v)

2.3 Experimental mice

2.3.1 Animal handling

Animals used for completion of this thesis were maintained and handled according to the Imperial College Subcommittee for Animal Research guidelines and the British Home Office regulations.

2.3.2 Transgenic mouse models

2.3.2.1 HP1 γ knockout (HP1 γ -KO) mice

HP1 γ -KO mice were generated by Naruse and colleagues with a gene trap method where a single gene-trap retroviral vector (ROSAN β -geo) was inserted into intron 1 (998bp downstream of exon 1) of the *Cbx3* gene (encoding HP1 γ) (Figure 2.1) (Naruse, Fukusumi et al. 2007). With the insertion of the gene trap vector, a fusion transcript containing HP1 γ exon 1 and β -geo of the gene-trap vector is generated and transcription terminated prematurely at the inserted polyadenylation site (pA). Hence, functional HP1 γ protein cannot be produced. HP1 γ -KO mice which were originally on a C57BL/6 background were back-crossed at least ten times on to the CBA/ca background.

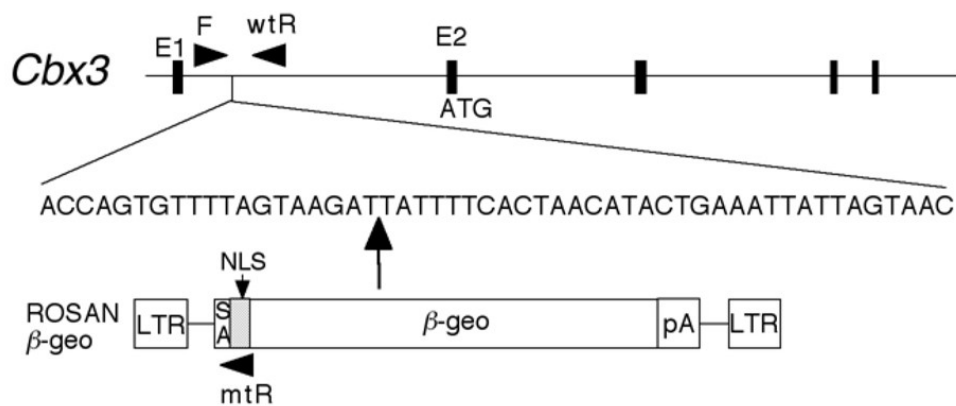


Figure 2.1 Schematic diagram demonstrating the insertion site of the gene trap vector in the *Cbx3* gene in the HP1 γ -KO mice. A single gene-trap retroviral vector (ROSAN β -geo) was inserted into intron 1 of the *Cbx3* gene. Insertion leads to generation of pre-mature non-functional transcript. Genotyping PCR primers are indicated: HP1 γ common forward (F), HP1 γ wild-type reverse (wtR), HP1 γ mutant reverse (mtR). This figure is adapted with permission from The Company of Biologists Ltd: Development “Takada, Y., C. Naruse, Y. Costa, T. Shirakawa, M. Tachibana, J. Sharif, F. Kezuka-Shiotani, D. Kakiuchi, H. Masumoto, Y. Shinkai, K. Ohbo, A. H. Peters, J. M. Turner, M. Asano and H. Koseki (2011). ‘HP1gamma links histone methylation marks to meiotic synapsis in mice.’ Development 138(19): 4207-4217.” Copyright (2011)

2.3.2.2 H3.3B-EGFP mice

H3.3B-EGFP mice containing knock-in construct of H3.3B-EGFP were generated by Dr. Vineet Sharma (Imperial College London, London, U.K.) and Dr. Buhe Nashun (MRC Clinical Science Centre, London, U.K.). H3.3B-EGFP mice were generated by inserting the EGFP gene downstream of the coding sequence (CDS) of the *H3f3b* gene (encoding histone variant H3.3b) (Figure 2.2). Hence, animals containing this target knock-in will produce H3.3B-EGFP fusion proteins.

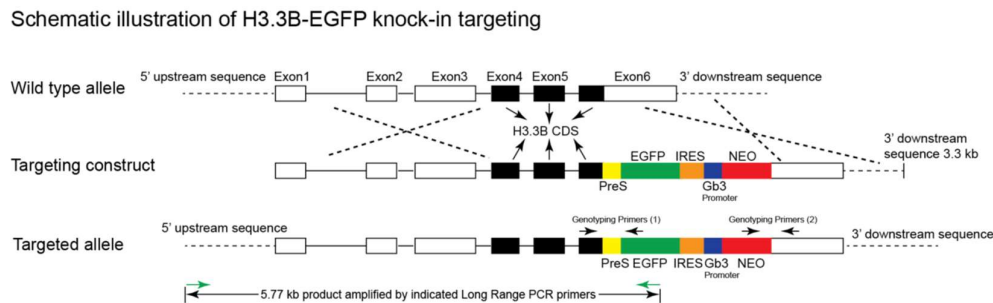


Figure 2.2 Schematic diagram demonstrating the H3.3B-EGFP knock-in construct in the H3.3B-EGFP mice. The EGFP construct was inserted downstream of the coding sequence of the H3.3B gene. Genotyping primers (2) were used for this thesis. This figure is reprinted from Molecular Cell, Nashun, B., P. W. Hill, S. A. Smallwood, G. Dharmalingam, R. Amouroux, S. J. Clark, V. Sharma, E. Ndjetehe, P. Pelczar, R. J. Festenstein, G. Kelsey and P. Hajkova “Continuous Histone Replacement by Hira Is Essential for Normal Transcriptional Regulation and De Novo DNA Methylation during Mouse Oogenesis.” Copyright (2015) with permission from Elsevier. (Nashun, Hill et al. 2015)

2.3.2.3 HP1 γ knockout H3.3B-EGFP (HP1 γ H3.3B-EGFP) mice

HP1 γ H3.3B-EGFP mice having heterozygous knockout of HP1 γ (HP1 γ ^{+/-}) and expressing H3.3B-EGFP fusion protein (H3.3B-EGFP ^{+/-}) were generated by mating the HP1 γ -KO mice (HP1 γ ^{+/-}) with the H3.3B-EGFP mice (H3.3B-EGFP ^{+/-}). By interbreeding these mice, E13.5 embryos which express the H3.3B-EGFP fusion protein and are either wild type (WT) (HP1 γ ^{+/+}, H3.3B-EGFP ^{+/-}) or homozygous knockout for HP1 γ (HP1 γ ^{-/-}, H3.3B-EGFP ^{+/-}) were generated. MEFs were subsequently derived from these embryos and used for experiments. Figure 2.3 illustrates the mating scheme of this line.

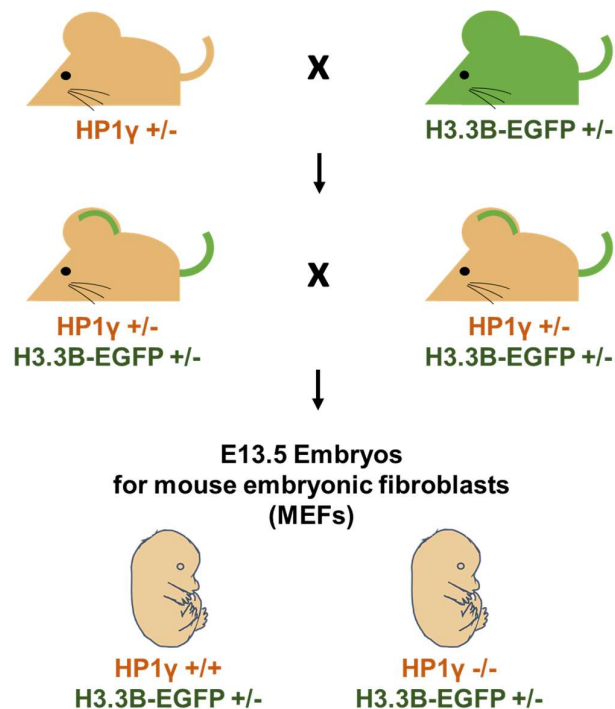


Figure 2.3 Schematic diagram demonstrating the mating scheme of the HP1 γ H3.3B-EGFP mice. Mice with heterozygous knockout of HP1 γ and expressing H3.3B-EGFP (HP1 γ ^{+/-}, H3.3B-EGFP ^{+/-}) were generated by mating the HP1 γ -KO mice (HP1 γ ^{+/-}) with the H3.3B-EGFP knock-in mice (H3.3B-EGFP ^{+/-}). By interbreeding these mice, E13.5 embryos with either WT or homozygous knockout HP1 γ which express H3.3B-EGFP fusion protein were generated and MEFs were then derived from these embryos.

2.3.2.4 HP1 γ heterozygous knockout mice with *hFXN* transgene (HP1 γ -KO *hFXN* mice)

HP1 γ -KO *hFXN* mice which are heterozygous knockout of HP1 γ and carrying *hFXN* transgene were generated by mating the HP1 γ -KO mice (HP1 γ ^{+/-}) with the *hFXN* mice which carry the pathological human frataxin transgene (*hFXN*). *hFXN* mice were previously generated by rescuing frataxin knockout mice with YAC FRDA transgenes which contain the entire *FXN* gene with (GAA)_n repeat expansions. More details of the *hFXN* mice can be found in “Rescue of the Friedreich’s ataxia knockout mouse by human YAC transgenesis” by Pook M. *et al* published in Neurogenetics (Pook, Al-Mahdawi et al. 2001). HP1 γ -KO *hFXN* mice were generated, maintained and genotyped by Dr. Mark Pook and colleagues (Brunel University London, United Kingdom).

2.4 Genotyping

For genotyping of transgenic mice, earclip samples were lysed in 200 μ l of lysis buffer (0.1M Tris HCl pH8.0, 5mM EDTA pH8.0, 0.2M NaCl, 0.2% SDS) supplemented freshly with proteinase K (200 μ g/ml) (Roche). For genotyping of MEFs, heads of embryos were lysed in 1ml of lysis buffer supplemented with proteinase K (200 μ g/ml). DNA from samples were extracted and purified with phenol/chloroform (section 2.5) and precipitated with ethanol. Precipitated DNA was resuspended in sterile water and used for PCR genotyping with BiotaqTM DNA polymerase (Bioline). PCR products were analyzed by running in DNA agarose gel electrophoresis. List of primers used can be found in section 2.21.1.

2.4.1 Genotyping PCR conditions

2.4.1.1 HP1 γ genotyping

This PCR was performed to determine whether the transgenic mice or embryos are WT, heterozygous or homozygous knockout of HP1 γ . PCR on HP1 γ WT mice will give a single band of 501bp in WT allele PCR and no band for the mutant allele PCR. Homozygous PCR on HP1 γ -KO mice will give a single band of 525bp for the mutant allele PCR and no band for the WT allele PCR. PCR on heterozygous HP1 γ -KO mice will have both 501bp and 525bp bands i.e. the WT and mutant allele PCR respectively. Examples of PCR results can be found in Figure S1 in appendix.

PCR reaction mix:

Component	WT allele PCR	Mutant allele PCR
10x NH ₄ reaction buffer	2.5 μ l	2.5 μ l
MgCl ₂ (50mM)	1.25 μ l	1.25 μ l
BSA (10mg/ml)	0.75 μ l	0.75 μ l
dNTPs (10mM)	0.5 μ l	0.5 μ l
HP1 γ common forward(10mM)	0.5 μ l	0.5 μ l
HP1 γ wild-type reverse (10mM)	0.5 μ l	0 μ l
HP1 γ mutant reverse (10mM)	0 μ l	0.5 μ l
DNA template	(50ng)	(50ng)
Biotaq™ DNA polymerase	0.2 μ l	0.2 μ l
Water	Up to 25 μ l	Up to 25 μ l
Total	25 μ l	25 μ l

PCR condition:

Cycles	Temperature	Time
1	96 °C	3 min
5	96 °C	15 sec
	70 °C (-1 °C/cycle)	15 sec
	72 °C	40 sec
30	96 °C	15 sec
	58 °C	15 sec
	72 °C	45 sec
1	72 °C	10 min
1	12 °C	hold

2.4.1.2 GFP-H3.3 genotyping

This PCR was performed to determine whether the transgenic mice contain H3.3B-EGFP knock-in constructs. PCR on DNA from mice with this knock-in construct will give a single band of 272bp whilst DNA from WT mice will give no band. Examples of PCR results can be found in Figure S1 in appendix.

PCR reaction mix:

Component	
10x NH ₄ reaction buffer	2.5µl
MgCl ₂ (50mM)	1.25µl
BSA (10mg/ml)	0.75µl
dNTPs (10mM)	0.5µl
GFP (F) (10mM)	0.5µl
GFP (R) (10mM)	0.5 µl
DNA template	(50ng)
Biotaq™ DNA polymerase	0.2µl
Water	Up to 25µl
Total	25µl

PCR condition:

Cycles	Temperature	Time
1	96 °C	5 min
35	96 °C	30 sec
	65 °C	30 sec
	72 °C	30 sec
	72 °C	10 min
1	12 °C	hold

2.4.1.3 Kdm5d genotyping for sex determination of embryos

In order to determine the sex of the embryos harvested for generation of MEFs in this thesis, PCR on the Y chromosome gene *Kdm5d* was performed. PCR on DNA from male mice will give a single band of 597bp while DNA from female mice will give no band. Examples of PCR results can be found in Figure S1 in appendix.

PCR reaction mix:

Component	
10x NH ₄ reaction buffer	2.5µl
MgCl ₂ (50mM)	1.25µl
BSA (10mg/ml)	0.75µl
dNTPs (10mM)	0.5µl
Kdm5d (F) (10mM)	0.5µl
Kdm5d (R) (10mM)	0.5 µl
DNA template	(25ng)
Biotaq™ DNA polymerase	0.2µl
Water	Up to 25µl
Total	25µl

PCR condition:

Cycles	Temperature	Time
1	96 °C	5 min
35	96 °C	30 sec
	60 °C	30 sec
	72 °C	15 sec
	72 °C	10 min
1	12 °C	hold

2.5 Phenol/Chloroform extraction and purification of DNA

DNA in aqueous solution was extracted and purified with equal volumes of phenol/chloroform solution (phenol: chloroform: isoamylalcohol (25: 24: 1)). Samples were mixed vigorously and centrifuged at 12,000 x g, 4°C for 15min. Upper aqueous layer was obtained and the DNA was precipitated by mixing with 1:10 volume of 3 M of sodium acetate (NaOAc) pH 5.2 and two volumes of absolute ethanol. The samples were then incubated at -20°C or -80°C for at least 1 hour and centrifuged at 12,000 x g 4°C for 30min. The precipitated DNA pellet was washed with 70% ethanol and centrifuged at 12,000 x g 4°C for 15min. The DNA pellet was air-dried and resuspended in DNase-free water. DNA concentration was estimated by NanoDrop® Spectrophotometer ND-1000 with ND-1000 v3.3.1 software.

2.6 Lentiviral-mediated short hairpin-RNA (shRNA) knockdown

pGIPZ shRNA lentiviral vectors (Open Biosystems) containing specific shRNA sequences were employed for knockdown experiments in human fibroblast cell lines and HeLa cells. All constructs were extracted and purified using HiSpeed® Plasmid Maxi kit (#12643, Qiagen) following manufacturer's protocol and verified by DNA sequencing prior to the experiment.

On day 0, HEK293T cells of 95% confluence in 10cm culture dishes were split 1:4 and incubated overnight at 37°C, 5% CO₂.

On day 1, transfection of HEK293T cells were performed by adding the transfection mix dropwise and incubated at 37°C, 5% CO₂ for 3 hours. The component of the transfection mix is shown below:

Component	Amount (μg)
pMD2.G (envelop plasmid)	2
psPAX2 (packaging plasmid)	8
pGIPZ shRNA construct with target insert	20
Poly-ethyleneimine (PEI) (linear MW25,000) (Polysciences)	90
HEPES buffered saline (HPS)	(Add up to total volume of 1ml)

Culture media were then discarded and replaced by 10ml fresh media. Transfected HEK293T cells were incubated at 37°C, 5% CO₂ overnight.

On day 2, the culture media of HEK293T cells were discarded and replaced by 10ml fresh media (DMEM for experiments on human fibroblasts and HeLa cells and incubated overnight at 37°C, 5% CO₂. The cells were incubated at 37°C, 5% CO₂ overnight. 1.7 x 10⁵ human fibroblast cells and 2 x 10⁵ HeLa cells were seeded in each well of the 6-well plate with 3ml medium and incubated overnight at 37°C, 5% CO₂.

On day 3, culture media containing specific lentivirus from the transfected HEK293T cells were obtained and filtered through 0.45μm cellulose acetate syringe filter. Polybrene (Sigma) was added to the viral supernatant to a final concentration of 8μg/ml and mixed well. Fresh media was then added to the transfected HEK293T cells and incubated at 37°C, 5% CO₂ for 3 hours. For lentiviral infection of human fibroblast cell lines and HeLa cells, 2.5ml of 2-fold diluted or undiluted viral supernatant was added to the cells in each well of the 6-well plate and incubated at 37°C, 5% CO₂ for 3 hours. Infection of cells were then repeated by replacing the viral supernatant from human fibroblasts or HeLa cells with the culture media from transfected HEK293T cells and incubated overnight at 37°C, 5% CO₂. Fresh media was then added to transfected HEK293T cells and incubated at 37°C, 5% CO₂ overnight.

On day 4, infection of cells was repeated once as described and the cells were subcultured according to density and recovered in fresh media. Puromycin (Sigma) selection of positive clones was performed on day 7 which were cultured in puromycin containing media until cell number was sufficient for experiments. Puromycin concentrations used are listed below:

Cell type	Puromycin concentration ($\mu\text{g/ml}$)
Human Fibroblast cell lines	2
HeLa	1

Target	Open Biosystem clone identifier	shRNA construct	shRNA sequences (5'->3')
<i>CBX3</i> (HP1 γ)	V2LHS_258350	shHP1 γ 1	GTGAACAGTGGTTAATAAATT A
<i>CBX3</i> (HP1 γ)	V3LHS_300370	shHP1 γ 2	TGAAGTGTCTCAAATTGTAT A
Scramble	(Cat.# RHS4349)	Scr	CTCTCGCTTGGGCGAGAGT AAGTA
Empty vector	(Cat.# RHS4346)	Em	(N/A)

Table 2.1. Sequences of shRNA used for knockdown experiment

pGIPZ sequencing primer: 5' GCATTAAAGCAGCGTATC 3'

Maps of plasmid constructs pMD2.G, psPAX2 and pGIPZ shRNA construct can be found in appendix (Figure S2 –Figure S4).

2.7 DNA sequencing

150-300ng of plasmid DNA was sequenced with the Applied Biosystems 3730xl DNA Analyzer (MRC CSC Genomics Laboratory). Sequences obtained were analyzed using SnapGene® (GSL Biotech) software.

2.8 Total RNA Extraction

Total RNA from MEFs, human fibroblast cell lines, HeLa cells and mouse cerebellum was extracted using TRIZOL® Reagent (Invitrogen) following the protocol provided.

0.5x 10⁶ MEF cells or 10⁶ human fibroblast cell lines and HeLa cells were used per RNA extraction reaction. Cells were pelleted by centrifugation at 300 x g, room temperature for 5min and lysed with 1ml TRIZOL® Reagent. For mouse cerebella, frozen tissue samples were homogenised using the IKA ultra-turrax T25 homogeniser at highest power in 1ml TRIZOL® Reagent. Samples were then centrifuged at 12,000 x g, 4 °C for 5min. The fatty top layer was removed and the clear homogenate solution was transferred to a fresh tube. Homogenised cell and tissue samples were incubated for 5min at room temperature. Afterwards, 200µl chloroform per 1ml TRIZOL® Reagent was added to each sample which was vigorously shaken for 15sec. Samples were incubated at room temperature for 5 min and centrifuged at 12,000 x g, 4 °C for 15min. The aqueous phase was transferred to a fresh tube and mixed with 500µl isopropanol per 1ml TRIZOL® Reagent. Samples were incubated at room temperature for 10min and centrifuged at 12,000 x g, 4 °C for 25min. Supernatant was discarded and 1ml 70% ethanol per 1ml TRIZOL® Reagent was added to the precipitated RNA pellet. Samples were mixed by vortex and centrifuged at 7500 x g, 4 °C for 15min. Supernatant was discarded and the RNA pellets were air-dried followed by DNase treatment with Ambion® DNA free kit (Invitrogen). It was performed by resuspending the air-dried RNA pellet in 40µl master mix containing 33µl RNase free water, 2µl SUPERase.IN™ RNase inhibitor (Thermo Fisher Scientific), 4µl 10x DNaseI buffer and 1µl DNaseI followed by subsequent incubation at 37°C for 30min. Reactions were then stopped by addition of 4µl of inactivation reagent to the samples and incubation at room temperature for 2min. Samples were centrifuged at 10,000 x g for 2min and 35µl supernatant which contained the isolated RNA was retained for subsequent analysis or otherwise stored at -80°C.

2.9 Complementary DNA (cDNA) synthesis and amplification

ThermoScript® kit (Invitrogen) was used to reverse transcribe the total RNA extracted (with TRIOZOL® Reagent) into complementary DNA (cDNA) using a non-gradient cycler PCR machine (Peltier Thermal Cycler (PTC-200, BIORAD)). 1-2 µg of RNA was incubated at 65°C for 5min with 50ng of random hexamer and 2µl of 10mM dNTP. 4µl of cDNA synthesis buffer (5x), 1µl of DTT (0.1M) and 1µl of ThermoScript™ reverse transcriptase (15U/µl) was added to the reaction and incubated at 50°C for 60min and then at 85°C for 5 min. cDNAs generated were then analysed using quantitative real time polymerase chain reaction (qRT-PCR) or stored at -20°C.

2.10 Chromatin Immunoprecipitation (ChIP)

1.8×10^6 cells were seeded in each 10cm tissue culture dish and incubated at 37°C, 5% CO₂ overnight to obtain about 2.5×10^6 cells per dish the next day. The culture medium per dish was adjusted to 10ml and the cells were fixed in 1% formaldehyde at room temperature for 10min. The formaldehyde fixation reaction was quenched with 125mM glycine for 5min at room temperature. The cells were washed three times by ice-cold PBS and incubated in 4ml swelling buffer at 4°C for 10min. Cells were collected by scraping and the nuclei were isolated using a dounce homogeniser with tight pestle (40 strokes). Nuclei were then collected by centrifuging at $3,000 \times g$ 4°C for 5min.

To generate chromatin, nuclei pellet generated from 10^7 cells was resuspended in 1ml sonication buffer and transferred to a 15ml polystyrene tube. The nuclei suspension was sonicated using a Bioruptor® (Diagenode) at high energy, 30sec on-30sec off, with probe for 20min. Sonicated material was then centrifuged at $16,000 \times g$ 4°C for 30min and the sheared chromatin in the supernatant was retained. 50µl chromatin sample was reverse crosslinked by adding 100µl water, 5µl proteinase K (10µg/µl) and 6µl 5M NaCl and incubated at 65°C for 4 hours.

Sonication efficiency was checked on agarose gel (see Figure S5 in appendix for representative sonication gel picture).

For immunoprecipitation, specific antibodies were added to 200µl chromatin aliquots (equivalent to 2×10^6 cells) and incubated in the Bioruptor® for 45min, at low energy, 30sec on - 30sec off. Samples were centrifuged at 12,000 x g, 4°C for 10min and the supernatant was added to 50µl Dynabeads® Protein G (Invitrogen) washed three times with sonication buffer and incubated overnight at 4°C.

The next day, beads with antibody-chromatin complex were washed one time with sonication buffer, wash buffer A, wash buffer B and twice with 1xTE buffer for 5min at 4°C with rotation. Elution of immune complexes and purification of DNA were performed using IPure Kit (Diagenode). In brief, 100µl of elution buffer (composed of 96.2% buffer A and 3.8% buffer B from kit) was added to washed beads and incubated at 65°C for at least 4h with continuous shaking. For the input sample, 10µl of chromatin samples were mixed with 90µl elution buffer prepared as stated above and incubated at 65°C for at least 4h with continuous shaking. Supernatant was obtained and mixed with 2µl of ‘carrier’ provided by the kit and 100µl of 100% isopropanol. 15µl of DNA-binding magnetic beads was then added to each sample and incubated at room temperature for 1h with rotation. The supernatant was discarded and beads/DNA complexes were washed once with 100µl wash buffer 1 and then wash buffer 2 for 5min at room temperature with rotation. Captured DNA was eluted by incubating with twice with 25µl buffer C from the kit at room temperature for 15min and giving sample with total volume of 50µl. Samples were then used for downstream applications (qRT-PCR or ChIP-sequencing library preparation), or stored at -20°C until further use. A list of antibodies used in ChIP can be found in section 2.22.1

ChIP on human primary lymphocytes was carried out as previously described (Chan, Torres et al. 2013).

2.11 Quantitative real-time polymerase chain reaction (qRT-PCR)

Quantitative real-time polymerase chain reaction (qRT-PCR) was used to analyse samples from ChIP experiments and check the expression level of genes after RNA extraction and cDNA synthesis. qRT-PCR reactions were prepared using SYBR® Green Jumpstart™ Taq ReadyMix™ (Sigma-Aldrich®) as follows and performed in Low 96-well White Multiplate® PCR plates™ (BIORAD).

PCR reaction mix:

Component	
SYBR®	10µl
Forward primer (10mM)	0.5µl
Reverse primer (10mM)	0.5µl
DNA template	Δ
Water	Up to 20µl
Total	20µl

PCR condition:

Cycles	Temperature	Time
1	95 °C	10 min
40	95 °C	30 sec
	58 °C	30 sec
	72 °C	30 sec
	80 °C	1 sec
(Plate read)	82 °C	1 sec
(Plate read)	85 °C	1 sec
Melting curve read from 70°C to 96 °C, read every 0.5 °C, hold 1 sec		

Full list of primers used for qRT-PCR can be found in section 2.21.2 and 2.21.3. The efficiency of all the primers used in this thesis were calculated by a standard curve generated by qRT-PCR on serial diluted DNA samples. Efficiency of primers in percentage were calculated by the formula:

$$\text{Primer efficiency (\%)} = (10^{(-1/\text{slope})} - 1) * 100$$

where the slope is the slope of the standard curve generated. This value represents the increased amount of PCR product after each PCR cycle. All primers used in this thesis have primer efficiency of 90-110%. (Representative curve see Figure S6 in appendix). For analysis of qRT-PCR data, $2^{-\Delta\Delta CT}$ method was used.

2.12 RNA sequencing library preparation and sequencing

RNA sequencing libraries were prepared using the TruSeq® RNA sample preparation kit v2 (Illumina®) following 'Low Sample (LS) protocol' provided by the manufacturer. 0.5µg of good quality RNA checked by Agilent 2100 Bioanalyzer (Agilent Technologies, Inc.) using the RNA 6000 Nano kit (#5067-1511, Agilent Technologies) was used per RNA-seq library preparation reaction. Prepared libraries were validated by Agilent 2100 Bioanalyzer using High Sensitivity DNA Analysis Kits (#5067-4626, Agilent Technologies, Inc.) following the manufacturer's protocol. Samples of sufficient quality were sequenced with HiSeq2500 (Illumina®). Representative Bioanalyzer results for good quality RNA and RNA sequencing libraries are shown in Figure S7 and Figure S8 in appendix.

2.13 RNA sequencing analysis

Raw data obtained from sequencing were processed using RTA (ver. 1.17.21.3) with default filter and quality settings from Illumina®. The reads were demultiplexed with CASAVA 1.8.2 (allowing 0 and 1 mismatch). Raw reads (fastq files) were aligned to the mouse genome (mm10) using Tophat aligner (tophat2 ver. 2.0.11) (Trapnell, Roberts et al. 2012). Aligned read counts data were generated by htseq work package (ver. 0.5.4p3). Differential expression analysis was performed using DESeq2 Bioconductor package (Love, Anders et al. 2014). Raw p-values were adjusted for multiple testing using the Benjamini-Hochberg procedure. All GO categories overrepresented with adjusted p-value of less than 0.05 were obtained. Gene ontology (GO) analysis was performed using the GOstats Bioconductor package (Falcon and Gentleman 2007). Statistical overrepresentation test was performed using the Panther classification system (<http://pantherdb.org/>) (Mi, Muruganujan et al. 2013). The mouse imprinted genes list was taken from World Wide Web Site - Mouse Imprinting Data and References - http://www.har.mrc.ac.uk/research/genomic_imprinting/ (link is external). Williamson CM, Blake A, Thomas S, Beechey CV, Hancock J, Cattanaach BM, and

Peters J (2013), MRC Harwell, Oxfordshire. Venn diagrams presented in this thesis were generated by Venny 2.0.2 (Oliveros, J.C. (2007-2015) Venny. An interactive tool for comparing lists with Venn's diagrams. (<http://bioinfogp.cnb.csic.es/tools/venny/index.htm>))

2.14 ChIP sequencing (ChIP-seq) library preparation and sequencing

ChIP-seq libraries were prepared using the NEBNext® Ultra™ DNA Library Prep Kit for Illumina® (#E7370, New England BioLabs®). Input and ChIP DNA were estimated by Quant-iT™ PicoGreen® dsDNA Assay (#11496, Invitrogen™). 8-10ng of DNA was obtained per ChIP-seq library preparation reaction. End repair reaction was prepared as follow:

End repair reaction mix:

Component of end repair reaction	
End Prep Enzyme Mix	3µl
End Repair Reaction Buffer (10X)	6.5µl
Input/ChIP DNA	(8-10ng)
Water	Δ
Total	65µl

End repair reaction samples were incubated at 20°C for 30min, 65°C for 30min and held at 4°C. Sequencing adaptor ligation was then performed as follow:

Adaptor ligation reaction mix:

Component of adaptor ligation	
End-repaired DNA sample	65µl
Blunt/TA Ligase Master Mix	15µl
NEBNext® Adaptor for Illumina® (1.5µM)*	2.5µl
Ligation Enhancer	1µl
Total	83.5µl

*NEBNext® Multiplex Oligos for Illumina® (#E7335, New England BioLabs®)

Samples were incubated at 20°C for 15min. 3µl of the USER™ Enzyme was added to each ligation reaction samples and incubated at 37°C for 15min.

To clean up adaptor-ligated DNA, 86.5µl of Agencourt AMPure®XP Beads (#A63881, Beckman Coulter) was added to each sample and incubated at room temperature for 5min. Supernatant was discarded and the beads were washed twice with 200µl freshly prepared 80% ethanol. Beads were air-dried for 5min and the bound-fraction was eluted in 20µl 0.1xTE.

Cleaned-up adaptor ligated DNA samples were enriched by PCR amplification as follows:

PCR amplification reaction mix:

Component	
Adaptor Ligated DNA Fragments	20µl
NEBNext® High Fidelity 2X PCR Master Mix	25µl
Index Primer*	2.5µl
Universal PCR Primer*	2.5µl
Total	50µl

*NEBNext® Multiplex Oligos for Illumina® (#E7335, New England BioLabs®)

PCR condition:

Cycles	Temperature	Time
1	98 °C	5 min
12	98 °C	10 sec
	65 °C	30 sec
	72 °C	30 sec
1	72 °C	5 min
1	4 °C	hold

Enriched DNA samples were resolved in 2% agarose gels (prepared with 0.5X TAE). DNA fragments of 200-400bp were extracted and cleaned up by a QIAquick® Gel extraction kit (#28706 Qiagen) following manufacturer's protocol. Prior to sequencing, the purified DNA fragments (ChIP sequencing library) were analysed with the Agilent 2100 Bioanalyzer using High Sensitivity DNA Analysis Kits (#5067-4626, Agilent Technologies, Inc.) following the manufacturer's protocol (see Figure S9 in appendix for representative Bioanalyzer result). Samples of sufficient quality were sequenced with HiSeq2500 (Illumina®).

2.15 ChIP sequencing analysis

ChIP-seq reads were aligned to the mouse genome version mm9 using bwa version 0.7.5a. Peak calling was performed using macs/1.4.1. Quality control plot and statistics were generated using ChIPQC package from Bioconductor (Carroll et al., in press). Feature visualisation and normalisations were carried out with soGGi package from Bioconductor (Dharmalingam and Carroll, 2015).

2.16 Immunofluorescence (IF)

MEFs cells were grown on coverslips (12mm diameter, 0.085 - 0.130mm thick) without coating. Coverslips with adhered cells were washed once in 1x PBS and fixed with 4% paraformaldehyde in PBS at room temperature for 10min. Coverslips were washed three times in 2x PBS and incubated in 30% glycerol in 1xPBS for 10min at room temperature. Coverslips in glycerol were then snap frozen in liquid nitrogen and stored at -80°C for later use.

For immunofluorescence, coverslips were thawed from -80°C and washed three times with 1xPBS. To permeabilise MEF cells on coverslips, 0.5% Triton-X 100 in PBS was added and incubated at room temperature for 30min. Fixed and permeabilised cells on coverslips were then washed three times with 1xPBS and incubated in 20mM glycine in PBS at room temperature for 30min. In order to block the non-specific antibody binding sites, coverslips were incubated in PBS+ (blocking solution) at room temperature for 1 hour. Subsequently, cells on coverslips were incubated in primary antibody pre-diluted in blocking solution and incubated in dark for 2 hours at room temperature. Cells on coverslips were washed with blocking solution and incubated with secondary antibody pre-diluted in blocking solution and incubated in the dark for 1 hour at room temperature. Cells on coverslips were washed with blocking solution and 1xPBS and incubated in DAPI (5ng/ul) pre-diluted in 1xPBS for 15min at room temperature. Finally, stained cells on coverslips were mounted with 6-7µl Vectashield mounting medium (Vector

Laboratories) and examined under a Leica confocal microscope or Leica widefield fluorescence microscope and analysed with Leica LAS AF Lite Software (Leica) or ImageJ (NIH, USA). The list of antibodies used in IF can be found in section 2.22.5 and 2.22.6. All the cells examined with immunofluorescence were in passage 1.

2.17 Co-immunoprecipitation (Co-IP)

6 x 10⁶ MEFs were used per co-immunoprecipitation experiment. Cells were pelleted at 300 x g, room temperature for 5min and washed once with 500µl buffer A followed by centrifugation at 300 x g, 4°C for 5 min. Supernatant was discarded and the cell pellets were lysed with 500µl buffer A containing 0.2% Triton X-100 and incubated on ice for 10 min. Samples were then centrifuged at 600 x g, 4°C for 5 min and the nuclei pellets were obtained. Nuclei were resuspended in 250µl of buffer A containing 2mM CaCl₂ and 1U of Micrococcal Nuclease (MNase) (Sigma) and incubated at 1400rpm, 4°C for 50 min. The reaction of MNase was stopped by addition of EGTA to a final concentration of 10mM. 5M NaCl was added to each sample to a final concentration of 340mM and the samples were incubated at 4°C for 60 min with gentle mixing. Samples were centrifuged at 8,000 x g, 4°C for 5min and the supernatants (nuclear extract) were obtained. Equilibration buffer was added to each sample to lower the salt concentration to 150mM NaCl.

For immunoprecipitation, specific antibody was mixed with nuclear lysate and 50µl Protein G Dynabeads® (Invitrogen) washed three times with buffer A. Samples were then incubated overnight at 4°C. Beads were then washed three times in 200µl wash buffer by vortexing. Immunoprecipitated material was eluted in 30µl SDS Sample buffer and boiled for 10min. Dithiothreitol (DTT) was then added to final concentration of 1mM and boiled again for 10min. Samples were then analysed by western blotting. Details of antibodies used for immunoprecipitation can be found in section 2.22.2.

2.18 Protein extraction

Unless otherwise stated, protein lysate used for experiments in this thesis was prepared using RIPA buffer (supplemented with protease inhibitors freshly before use) and the protein concentration was estimated using a bovine serum albumin (BSA) dilution standard curve with the Bradford Protein Assay (BIO-RAD). Prior to loading into the gel for western blotting, protein sample were mixed 1:1 with 2x Laemmli buffer and denatured by heating at 100°C for 10min. Protein samples were stored at -20°C.

2.19 Frataxin protein measurement using Quantity Dipstick Assay Kit

Frataxin Protein Quantity Dipstick Assay Kit (ab109881, Abcam®) was used to measure Frataxin protein level in healthy and FRDA human fibroblast cell lines. About 10⁶ cells were pelleted and resuspended in 200µl buffer A provided by the kit. Samples were incubated on ice for 20min and centrifuged at 16000 x g, 4°C for 20min. Supernatant (protein lysate) was retained and the protein concentration of the samples was estimated using a BSA standard curve with the bicinchoninic acid (BCA) assay kit (Sigma®) according to manufacturer's protocol. 25µl of protein samples at 1.6µg/µl was mixed with 25µl 2x buffer B and added to the microplate well provided, incubated at room temperature for 5min and followed by gently vortex in order to dissolve the dried gold-conjugated secondary antibody in each well of the microplate. Dipsticks were then put carefully onto each well and let to soak in the entire protein sample at room temperature. To wash the dipstick, 30µl of buffer C was added to each well letting the wash soak up completely onto the dipstick by capillary action. The dipsticks were then air-dried at room temperature for 20min and the signal intensity of the bands corresponding to Frataxin protein were measured by the Mitosciences MS1000® dipstick reader. Frataxin protein concentration in each sample was estimated using a recombinant Frataxin standard curve (Figure S10 in appendix).

2.20 Western blotting

10-30µg of denatured protein samples were resolved in denaturing SDS-PAGE gels prepared as follows using the BIO-RAD Mini-PROTEAN® Tetra Systems (BIO-RAD) with 1mm plate separation.

	Resolving gel (12.5%)	Stacking gel (4%)
Water	3.125ml	6ml
30% Acrylamide	4.17ml	1.33ml
1.5M Tris pH8.8	2.5ml	0ml
0.5M Tris pH6.8	0ml	2.5ml
10% SDS	100µl	100µl
10% ammonium persulphate (APS)	100µl	100µl
Tetramethylethylenediamine (TEMED)	10µl	10µl
Total	10ml	10ml

Protein samples were resolved by running the SDS-PAGE gel at 150V for 70-90min. After electrophoresis was completed, the SDS-PAGE gel was then incubated in 1x transfer buffer. The transfer stack was prepared by sandwiching the SDS-PAGE gel and the Hybond-N+ PVDF membrane (GE Healthcare) (activated by incubating in 100% methanol for about 2min and subsequently rinsed with 1x transfer buffer) with 3 pieces of Whatman 3MM filter papers on either side and placed into the Mini Trans-Blot® system (BIO-RAD) with the PVDF membrane and the SDS-PAGE gel facing the negative and positive electrode respectively. Protein in the SDS-PAGE gel was transferred to the membrane at 100V, 4°C for 1h. After transfer, the membrane was blocked for 1 hour at room temperature with 5% milk (in PBST/TBST) Primary antibody diluted in 5% milk or 5% BSA (in PBST/TBST) was added to the membrane and incubated at room temperature for 2 hours or 4°C overnight. The membrane was then washed three times for 10min at room temperature with PBST or TBST and incubated with horseradish peroxidase (HRP)-conjugated secondary antibody diluted in 5% milk (in PBST/TBST) for 1 hour at room temperature. Next, the membrane was washed three times with PBST or TBST for 10min at room temperature and the blot was developed using Amersham ECL Prime Western Blotting Detection Reagent (GE Healthcare) following the manufacturer's protocol in the darkroom. A list of antibodies used in western blotting can be found in section 2.22.3 and section 2.22.4.

2.21 Lists of primers

2.21.1 List of primers used for genotyping:

Primer name	Purpose	Primer sequences (5'-3')
HP1 γ common forward	HP1 γ genotyping	GAGTGATTACCGACACCACCA
HP1 γ wild-type reverse	HP1 γ genotyping	TTTAATCGGAGACTTGAAGAGC
HP1 γ mutant reverse	HP1 γ genotyping	GTTCGCTTCTCGCTTCTGTT
GFP (F)	H3.3B-EGFP genotyping	GACCGCTTCCTCGTGCTTTA
GFP (R)	H3.3B-EGFP genotyping	GAGCCACAGTGCTCACATCA
Kdm5d (F)	Sex determination	ACAAAGTGGGGGCAAAAAGT
Kdm5d (R)	Sex determination	AGTTATGACCCTCACCACAAGA

2.21.2 List of primers used for qRT-PCR checking gene expression level:

Primer name	Gene target	Species	Primer sequences (5'-3')
human β -ac (F)	ACTB (β -actin)	Human	GCGGGAAATCGTGCGTGACAT
human β -ac (R)	ACTB (β -actin)	Human	GATGGAGTTGAAGGTAGTTTCGTG
AL-sat (F)	α -Satellite	Human	AACTCACAGAGTTGAACGATCC
AL-sat (R)	α -Satellite	Human	ACCTCAAAGCGGCTGAAAT
m_HP1beta (F)2	<i>Cbx1</i> (HP1 β)	Mouse	GCCCTGACCTTATTGCTGAG
m_HP1beta (R)2	<i>Cbx1</i> (HP1 β)	Mouse	CCTCGTGGCTTTTCTGACTC
HP1 γ 1435	<i>Cbx3</i> (HP1 γ)	Mouse	GGTCCAGGTCAGCCAGTCTA
HP1 γ 1554	<i>Cbx3</i> (HP1 γ)	Mouse	CCAGCCACGATTCTATTTCC
h_HP1gamma (F)	<i>CBX3</i> (HP1 γ)	Human	GCTGGCAAAGAAAAAGATGGTAC
h_HP1gamma (R)	<i>CBX3</i> (HP1 γ)	Human	CATCAATTCTCCACTGCTGTCTGT
m_HP1alpha (F)	<i>Cbx5</i> (HP1 α)	Mouse	GGAAATCCAGTTTCTCCAACA
m_HP1alpha (R)	<i>Cbx5</i> (HP1 α)	Mouse	GCTCCGATGATCTTTTCTG
FXN mRNA (F)	<i>FXN</i> (Frataxin)	Human	ATGTCTCCTTTGGGAGTGGTGTCT
FXN mRNA (F)	<i>FXN</i>	Human	CCCAGTCCAGTCATAACGCTTA

GSATII (F)	(Frataxin) γ-Satellite(II)	Human	GGT ACGAAAGTGCCTTCCCATC
GSATII (R)	γ - Satellite(II)	Human	CACCTCCCCTCCCTCAAAC
Mj-sat (F)	Major-Satellite	Mouse	GACGACTTGAAAAATGACGAA ATC
Mj-sat (R)	Major-Satellite	Mouse	CATATTCCAGGTCCTTCAGTGT GC
18S (F)	Rn18s (18S rRNA)	Mouse	ATGGTAGTCGCCGTGCCTAC
18S (R)	Rn18s (18S rRNA)	Mouse	CCGGAATCGAACCCTGATT

2.21.3 List of primers used for ChIP-qRT-PCR:

Primer name	Gene target	Species	Primer sequences (5'-3')
EF1α (F)	<i>Eef1a1</i>	Mouse	CGGGTACCGGGATTATGGGTTATG AGAA
EF1α (R)	<i>Eef1a1</i>	Mouse	TAGGGATAGATTCTGGCCGCCCTC TTG
Gapdh+4K(F)	<i>Gapdh</i>	Mouse	GAGCCCTCCCTACTCTCTTGAAT
Gapdh+4K(R)	<i>Gapdh</i>	Mouse	ACACCGCATTAACCAAGGA
Gapdh(F)	<i>Gapdh</i>	Mouse	AGTCCGTATTTATAGGAACCCGGAT GGTGG
Gapdh(R)	<i>Gapdh</i>	Mouse	ATGAGAGAGGCCCGAGCTACTCGCG GCTTTA
Mj-sat (F)	Major-Satellite	Mouse	GACGACTTGAAAAATGACGAAATC
Mj-sat (R)	Major-Satellite	Mouse	CATATTCCAGGTCCTTCAGTGTGC
Myl3 (F)	<i>Myl3</i>	Mouse	TGCCGTCAGCTAGAGTCTGCTGTG TCAAGG
Myl3 (R)	<i>Myl3</i>	Mouse	GTATTGAGAAAGAGAAAAGTGGG GGAGGGG
Neurod6(F)	<i>Neurod6</i>	Mouse	TTTTTCCCTATCAGTCTAACCTCCT GTGTTGA
Neurod6(R)	<i>Neurod6</i>	Mouse	AAAAGTGACATTGATGCCAACTGC CAGAGC

2.22 Lists of antibodies

2.22.1 List of antibodies for chromatin immunoprecipitation (ChIP):

Antibody	Type/Isotype	Dilutions	Company	Catalogue no.
Anti-GFP	Rabbit polyclonal, IgG	10µg/IP	Abcam	Ab290
Anti-H3	Rabbit polyclonal, IgG	6ug/IP	Abcam	Ab1791
Anti-H3 (acetyl K9+K14+K18+K23+K27)	Rabbit polyclonal, IgG	8µg/IP	Abcam	Ab47915
Anti-H3.3	Rabbit polyclonal	10µg/IP	Millipore	09-838
Anti-H3K9me3	Rabbit polyclonal, IgG	6µg/IP	Millipore	07-442
Anti-H3K27me3	Mouse monoclonal IgG3	8µg/IP	Abcam	Ab6002
Anti-H4 (acetyl K5+K8+K12+K16)	Rabbit polyclonal IgG	8µg/IP	Millipore	06-866
Anti-HP1γ	Mouse monoclonal, IgG1	8µg/IP	Millipore	05-690

2.22.2 List of antibodies for co-immunoprecipitation (co-IP):

Antibody	Type/Isotype	Dilutions	Company	Catalogue no.
Anti-GFP	Rabbit polyclonal, IgG	10µg/IP	Abcam	Ab290
Anti-GFP	Mouse monoclonal, IgG1	5µg/IP	Abcam	Ab1218
Anti-HP1γ	Rabbit polyclonal, IgG	5µg/IP	Abcam	Ab10480
Mouse IgG	Normal mouse IgG	5µg/IP	Santa-cruz	sc-2025
Rabbit IgG	Normal rabbit IgG	5µg/IP	Millipore	PP64B

2.22.3 List of primary antibodies for western blot:

Antibody	Type/Isotype	Dilutions	Company	Catalogue no.
Anti- α -tubulin	Mouse monoclonal, IgG1	1:10000	Sigma	T5168
Anti-GFP	Rabbit polyclonal, IgG	1:1000	Abcam	Ab290
Anti-H3	Rabbit polyclonal, IgG	1:1500	Abcam	Ab1791
Anti-H3.3*	Rabbit polyclonal	1:500	Millipore	09-838
Anti-H3K9me3*	Rabbit polyclonal, IgG	1:1000	Abcam	Ab8898
Anti-HP1 α	Mouse monoclonal, IgG1	1:250	Millipore	MAB3446
Anti-HP1 β	Mouse monoclonal, IgG1	1:500	Millipore	MAB3448
Anti-HP1 γ	Mouse monoclonal, IgG1	1:5000	Millipore	MAB3450

*Antibodies that require the use of BSA

2.22.4 List of Secondary antibodies for western blot:

Antibody	Dilutions	Company	Catalogue no.
Goat anti-Mouse IgG, HRP-conjugated	1:20000	Life Technologies	G21040
Goat anti-Rabbit IgG, HRP-conjugated	1:20000	Santa-cruz	sc-2004
Goat anti-Rabbit IgG, HRP-conjugated	1:20000	Pierce	1858415

2.22.5 List of primary antibodies for immunofluorescence (IF):

Antibody	Type/Isotype	Dilutions	Company	Catalogue no.
Anti-GFP	Rabbit polyclonal, IgG	1:5000	Abcam	Ab290
Anti-H3K9me3	Rabbit polyclonal, IgG	1:50	Millipore	07-442
Anti-HP1 α	Mouse monoclonal, IgG1	1:50	Millipore	05-689
Anti-HP1 β	Mouse monoclonal, IgG1	1:50	Millipore	MAB3448
Anti-HP1 γ	Mouse monoclonal, IgG1	1:5000	Millipore	MAB3450

2.22.6 List of secondary antibodies for immunofluorescence (IF):

Antibody	Dilutions	Company	Catalogue no.
Goat anti-Mouse IgG, Alexa Fluor® 488 conjugated	1:600	Life Technologies	A11029
Dockey anti-Mouse IgG, Alexa Fluor® 568 conjugated	1:600	Life Technologies	A10037
Goat anti-Rabbit IgG, Alexa Fluor® 488 conjugated	1:600	Life Technologies	A11008
Goat anti-Rabbit IgG, Alexa Fluor® 568 conjugated	1:600	Life Technologies	A11036

2.23 Solutions

0.5x TAE - 20 mM Tris acetate, 0.5 mM EDTA

10x TE - 100 mM Tris-HCl, 10 mM EDTA

5x TBE - 450 mM Trisborate, 10 mM EDTA

Buffer A (co-immunoprecipitation) - 20mM HEPES, pH 7.9, 10mM KCl, 1.5mM MgCl₂, 0.34M sucrose, 10% glycerol supplemented with 1mM dithiothreitol (DTT), 1 mM PMSF and 0.5µl of protease inhibitor cocktail per 1ml buffer freshly

Equilibration buffer (co-immunoprecipitation) - 20mM HEPES, pH7.9, 1.5mM MgCl₂, 0.2mM EGTA, 25% glycerol. Supplemented with 0.5 mM PMSF, 0.5µl of protease inhibitor cocktail per 1ml buffer freshly

HEPES buffered saline (HPS) - 137mM NaCl, 5.1mM KCl, 0.7mM Na₂PO₄, 21mM HEPES, 0.1% (w/v) glucose, pH7.05

Laemmli buffer (2X) (western blot) - 4% SDS, 10% 2-mercaptoethanol, 20% glycerol, 0.004% bromophenol blue, 0.125 M Tris HCl

LB - 1% tryptone, 0.5% yeast extract, 1% NaCl, adjust pH to 7.0-7.5 by NaOH

LB agar - 1% tryptone, 0.5% yeast extract, 1% NaCl, adjust pH to 7.0-7.5 by NaOH, 1.5% agar

Lysis buffer (genotyping) - 0.1M Tris HCl pH8.0, 5mM EDTA pH8.0, 0.2M NaCl, 0.2% SDS

Phenol/chloroform solution - Prepared by mixing 25:24:1 Phenol:chloroform:isoamylalcohol and equilibrated with 10 mM Tris-HCl, pH 8.0

Phenylmethanesulfonyl Fluoride (PMSF) (0.1M) - Prepared in isopropanol

Phosphate buffered saline (PBS) - 171mM NaCl, 3.3mM KCl, 10.1mM Na₂PO₄, 1.8 mM KH₂PO₄, pH7.4

Phosphate buffered saline with Tween-20 (PBST) (western blot) - 171mM NaCl, 3.3mM KCl, 10.1mM Na₂PO₄, 1.8 mM KH₂PO₄, 0.1% Tween-20, pH7.4

RIPA (Radio Immuno Precipitation Assay buffer) buffer (protein extraction) - 150 mM NaCl, 1.0% NP-40 or Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris, pH 8.0. Supplemented with 0.5 mM PMSF, 0.5µl of protease inhibitor cocktail per 1ml buffer freshly

Running buffer (western blot) - 25 mM Tris base, 190 mM glycine, 0.1% SDS, pH 8.3.

SDS Sample buffer (co-immunoprecipitation) - 100 mM Tris, pH 6.8, 4% SDS, and 20% glycerol

Sonication buffer (chromatin immunoprecipitation) - 50 mM HEPES pH 7.9, 140 mM NaCl, 1mM EDTA, 1% Triton X-100, 0.1% Sodium deoxycholate, 0.1% SDS, pH 7.9. Supplemented with 0.5 mM PMSF, 0.5µl of protease inhibitor cocktail per 1ml buffer freshly

Swelling buffer (chromatin immunoprecipitation) - 25 mM HEPES pH 7.9, 1.5 mM MgCl₂, 10 mM KCl, 0.1% NP-40, pH 7.9. Supplemented with 0.5 mM PMSF, 0.5µl of protease inhibitor cocktail per 1ml buffer freshly

TBST (western blot) - 50 mM Tris.HCl, pH 7.4 and 150 mM NaCl, 0.1% Tween-20, pH7.5

Transfer buffer (western blot) - 25 mM Tris base, 190 mM glycine, 20% methanol, pH 8.3

Wash buffer (co-immunoprecipitation) - 20mM HEPES, pH7.9, 150mM NaCl, 1.5mM MgCl₂, 0.2mM EGTA, 0,2% Triton X-100, 10% glycerol. Supplemented with 0.5 mM PMSF, 0.5µl of protease inhibitor cocktail per 1ml buffer freshly

Wash buffer A (chromatin immunoprecipitation) - 50 mM HEPES pH 7.9, 500 mM NaCl, 1mM EDTA, 1% Triton X-100, 0.1% Sodium-deoxycholate, 0.1% SDS, pH 7.9

Wash buffer B (chromatin immunoprecipitation) - 20 mM Tris pH 8.0, 1 mM EDTA, 250 mM LiCl, 0.5% NP-40, 0.5% Sodium-deoxycholate, pH8.0

Chapter 3 – Results

3.1 Investigation of the role of HP1 γ in gene regulation

HP1 γ is a member of the mammalian HP1 protein family. Notably, unlike HP1 α and HP1 β , it is found in both euchromatic and heterochromatic regions in interphase nuclei (Horsley, Hutchings et al. 1996, Minc, Allory et al. 1999, Nielsen, Ortiz et al. 1999, Minc, Courvalin et al. 2000). Perhaps surprisingly, this protein is thought to be involved in both the activation and repression of genes (see introduction section 1.4.2). Here, the role of HP1 γ in gene regulation was studied systematically by transcriptomic analysis of cells with or without HP1 γ . This study sought to provide better insight into the role of HP1 γ in the regulation of gene expression and associated physiology.

3.1.1 Mouse Embryonic fibroblasts (MEFs) are a good model for studying the role of HP1 γ in gene regulation

As mentioned in the introduction section 1.4.2, deletion of HP1 γ is associated with neonatal lethality in mice when bred on a pure genetic background (Naruse, Fukusumi et al. 2007, Takada, Naruse et al. 2011). Furthermore, even with a mixed genetic background, only a small percentage of mice with HP1 γ deletion were able to develop to adulthood (Brown, Bullwinkel et al. 2010, Takada, Naruse et al. 2011). Although the heterozygous HP1 γ knockout mice with one *Cbx3* gene (encoding HP1 γ) disrupted show no phenotype and develop into adulthood, variation of HP1 γ mRNA level was observed in the thymocytes obtained from these mice (Figure S11 in appendix). Thus, study of alternative models with complete HP1 γ deficiency would be likely to reveal more profound effects.

In this project, mouse embryonic fibroblasts (MEFs) derived from E13.5 embryos from a cross between heterozygous HP1 γ -KO mice were employed (see methods and material section 2.3.2.1). There is a 25% chance of getting wild type (WT) or homozygous knockout (HOM) embryos from this cross and the HP1 γ WT and

HOM MEFs derived from these embryos were used for experiments. The expression level of HP1 γ mRNA and protein were checked by qRT-PCR and western blot. Figure 3.1 shows that HP1 γ mRNA levels were significantly reduced in HP1 γ HOM MEFs, showing only 1.5% of that of the WT MEFs. Moreover, these cells showed undetectable level of HP1 γ protein (Figure 3.1). Diminished level of HP1 γ mRNA and protein in HP1 γ HOM MEFs make it a good model for studying the loss of function of HP1 γ .

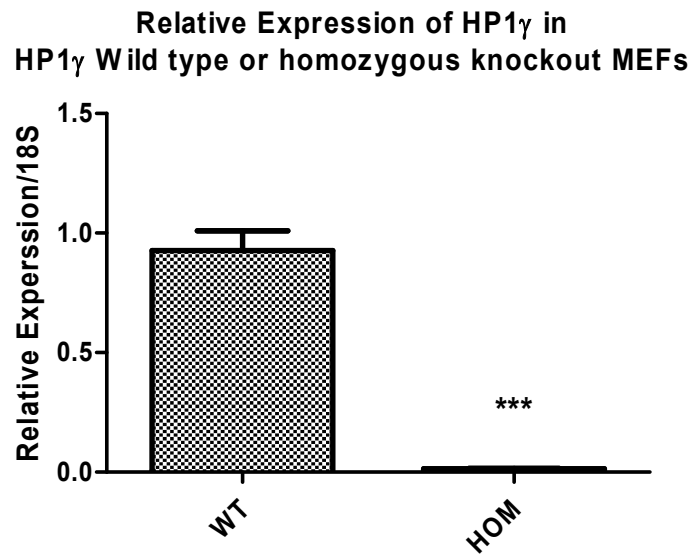
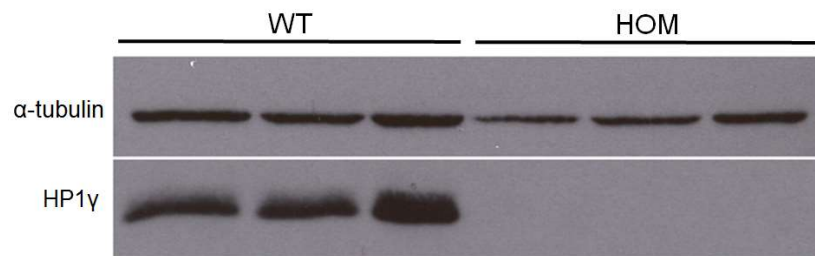
A**B**

Figure 3.1 Expression of HP1 γ in Mouse Embryonic fibroblasts (MEFs) derived from HP1 γ homozygous knockout embryos. (A) Relative expression of HP1 γ mRNA in HP1 γ wild type (WT) and HP1 γ homozygous knockout (HOM) mouse embryonic fibroblasts (MEFs). HP1 γ HOM MEFs showed 1.5% of HP1 γ mRNA comparing to WT MEFs. Student's t-test: ***P<0.0001 compared to WT after being normalised against 18S expression. Error bars: SEM, n $\geq$ 16. (B) Western blot analysis showing HP1 γ protein level in HP1 γ WT and HOM MEFs. HP1 γ HOM MEFs showed undetectable HP1 γ protein level. Alpha-tubulin (α -tubulin) was used as loading control. Western blots presented here are 3 representative experiments from MEFs derived from three 3 individual embryos.

3.1.2 HP1 γ is implicated in both activating and repressing gene expression and the magnitude of effect is sex dependent

In order to reveal the effect of HP1 γ on global gene expression patterns *in vivo*, high-throughput sequencing was performed on the total RNA extracted (RNA-seq) from HP1 γ WT and HOM MEFs. In this experiment, RNA extracted from three WT and three HOM MEF lines from both genders were analysed.

Differentially expressed genes in response to HP1 γ knockout were identified and the sample-to-sample distance (similarity) between technical and biological replicates from HP1 γ WT and HOM MEFs were explored for both male and female by clustering analysis (Figure 3.2A and B). The results are displayed as the dendrogram in Figure 3.2A. The technical replicates (obtained from two individual sequencing reactions) from each sample showed high similarity whereby they found clustered together as shown by the dendrogram. Also, the high degree of similarity between replicates were indicated by the dark blue colour in the heat map which depicts a sample-to-sample distance matrix where low distance (high similarity) is represented by dark blue. Although the three male HP1 γ WT and HOM samples were found in well-defined clusters according to their genotype, there was clearly variation between biological replicates which was greater in the HP1 γ HOM samples than the WT samples. The cells were morphologically indistinguishable and had the characteristic appearance expected of fibroblasts which argues against clonal outgrowth as in explanation of the variation between biological replicates. In contrast, although high similarity between technical replicates was also observed, the female biological replicates of the same genotypes did not cluster together well. For example, the WT sample 'WT1Frep1 and WT1Frep2' clustered together with the HOM sample 'HOM1Frep1 and HOM1Frep2' as indicated by the dendrogram and the blue colour in the heat map. Also, the dendrogram of the female samples does not show clear branches to distinguish the WT and HOM samples. This data indicates that this experiment is technically sound with high similarity observed between technical replicates. The RNA-seq data obtained from male HP1 γ WT and HOM MEFs show a high degree of similarity and is consistent between members within the same genotype group. However, relatively poor distinction between WT and HOM samples within females was revealed. Taken together, this result suggests

that the transcriptome male samples of the same genotype have a higher similarity compared to that of the female. This analysis suggests that HP1 γ deficiency has a more profound effect in males than females.

Indeed, our RNA-seq data revealed a much higher number of differentially expressed genes in male MEFs comparing to female MEFs. In response to HP1 γ knockout, a total of 4293 (18.3% of total) and 1187 genes (5.1% of total) were differentially expressed with P -value <0.05 in males and females respectively. In male MEFs, 2645 genes (61.6% of total number of dysregulated genes) were upregulated and 1648 genes (38.4% of total number of dysregulated genes) were downregulated (Figure 3.3A) while only 1009 genes (85.1% of total number of dysregulated genes) were upregulated and 177 genes (14.9% of total number of dysregulated genes) were downregulated in female MEFs by at least 1.5 fold (Figure 3.3B). Also, the downregulated genes in female showed a smaller magnitude of fold change when compared to male. Importantly, the observed difference is unlikely to be a consequence of the difference in HP1 γ expression level as no significant difference was observed between HP1 γ mRNA level in WT male and female MEFs examined by qRT-PCR (Figure S12 in appendix). Interestingly, there was a higher number as well as a bigger degree of change of upregulated genes than downregulated genes revealed in both males and females suggesting that HP1 γ may act more often as a silencer in the genome. This result indicates that HP1 γ indeed plays a role in both activating as well as repressing gene expression in our cell model. Also, the different magnitude of effect of HP1 γ on gene regulation observed in the two genders suggests that males have a higher dependency on HP1 γ in maintaining their normal gene expression profile.

In order to further investigate the similarities between male and female samples from each genotype, the top 30 expressed genes were sampled and the similarity in expression level of these genes were compared between the biological replicates. Heat maps were constructed to demonstrate the changes in expression level of these top 30 expression genes upon HP1 γ knockout. Consistent with the clustering analysis presented above (Figure 3.2), high similarity was observed between technical replicates for both male and female samples as indicated by their similar colours on the heat maps (Figure 3.4A and B). Male samples of the same genotypes

show a very similar colour on the heat map for most of the genes tested indicating their high degree of similarity (Figure 3.4A). A number of differentially expressed genes were also revealed here. In particular, a strong effect was observed on the *Igf2* gene which is subject to genomic imprinting. As shown in Figure 3.4A, all of the male WT MEF samples showed a higher expression level of the *Igf2* gene (as indicated by dark blue colour) relative to HP1 γ HOM MEFs (as indicated by pale green to white colour). Thus, deficiency in HP1 γ results in repression of *Igf2* gene (see below section 3.1.7). This data indicates a high consistency between male samples with the same genotype.

Female samples of the same genotype also showed similar colour on the heat map for most of the sampled genes. However, the HP1 γ HOM sample HOM3Frep1 and HOM3Frep2 showed different levels of expression of many of the genes when compared to the other two samples of the same genotype (Figure 3.4B). This particular sample was previously shown, in clustering analysis, to be furthest away from the wild type cluster (Figure 3.2B). For example, the expression of the *Igf2* gene which is dramatically downregulated in male HP1 γ HOM MEFs (Figure 3.4A) was found to be variable in female samples (Figure 3.4B). Thus, the HOM3Frep1 and HOM3Frep2, unlike the other female samples showed very low *Igf2* expression compared to WT. This low level of *Igf2* was similar to the previous observation in HP1 γ HOM males (Figure 3.4A and B). In fact, more variation in *Igf2* gene expression was observed in the WT female samples. For example, WT1Frep1 and WT1Frep2 showed higher *Igf2* expression when compared to WT3Frep1 and WT3Frep2. Taken together, these data suggest that the expression profile between male MEFs of the same genotype is more consistent than females. Although a bigger effect of HP1 γ was observed in male MEFs upon HP1 γ knockout, certain females may also rely heavily on HP1 γ for maintaining the expression level of certain genes.

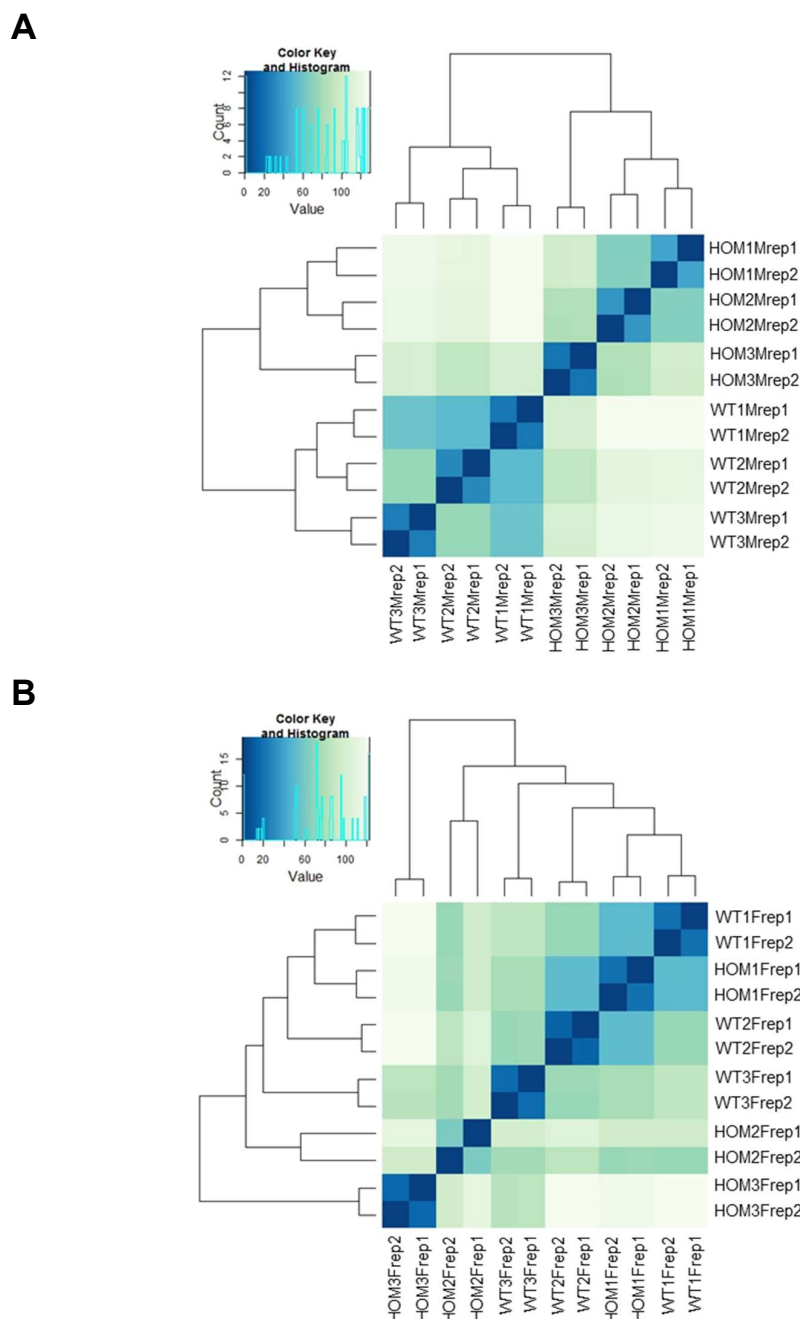


Figure 3.2 Clustering analysis of RNA-seq samples looking at similarities between samples. Sample clustering for RNA-seq data obtained from male (A) and female (B) HP1 γ WT and HOM MEFs. Variance-stabilising transformation was applied to obtain this data. Dark blue colour of heat map represents zero (highly similar) distance while pale green colour represents a large distance (dissimilar). (A) Clustering analysis of samples from males HP1 γ WT and HOM MEFs showed good clustering between technical replicates as well as biological replicates of the same genotype indicating the high similarity of these samples. Male WT samples and their technical replicates are: WT1Mrep1 and WT1Mrep2; WT2rep1 and WT2rep2; WT3rep1 and WT3rep2. Male HOM samples and their technical replicates are:

HOM1Mrep1 and HOM1Mrep2; HOM2Mrep1 and HOM2Mrep2; HOM3Mrep1 and HOM3Mrep2. (B) Female samples showed good clustering between technical replicates, however, the biological replicates did not. Female WT samples and their technical replicates are: WT1Frep1 and WT1Frep2; WT2Frep1 and WT2Frep2; WT3Frep1 and WT3Frep2. Female HOM samples and their technical replicates are: HOM1Frep1 and HOM1Frep2; HOM2Frep1 and HOM2Frep2; HOM3Frep1 and HOM3Frep2.

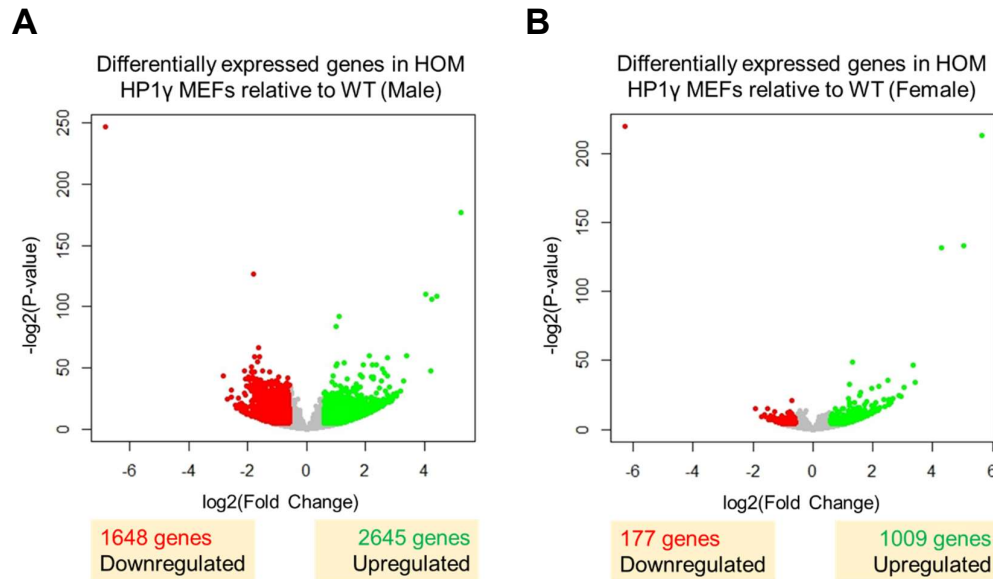


Figure 3.3 Volcano plots demonstrating the differentially expressed genes in male and female HP1 γ HOM MEFs relative to WT. Red and green dots represent genes that were down- or up-regulated by at least 1.5 fold respectively. Grey dots represent genes that are not significantly changed. X axes of these plots represent the log₂ fold change of the differentially expressed genes while Y axes represent the -log₂ P-value. (A) Male HP1 γ HOM MEFs showed 2645 upregulated genes and 1648 downregulated genes which were differentially expressed by at least 1.5 fold relative to WT. (B) The effect of HP1 γ knockout in female was much smaller compared to male. In female HP1 γ HOM MEFs, only 1009 genes and 177 genes were up- or downregulated by at least 1.5 fold respectively. P-value<0.05, n=3.

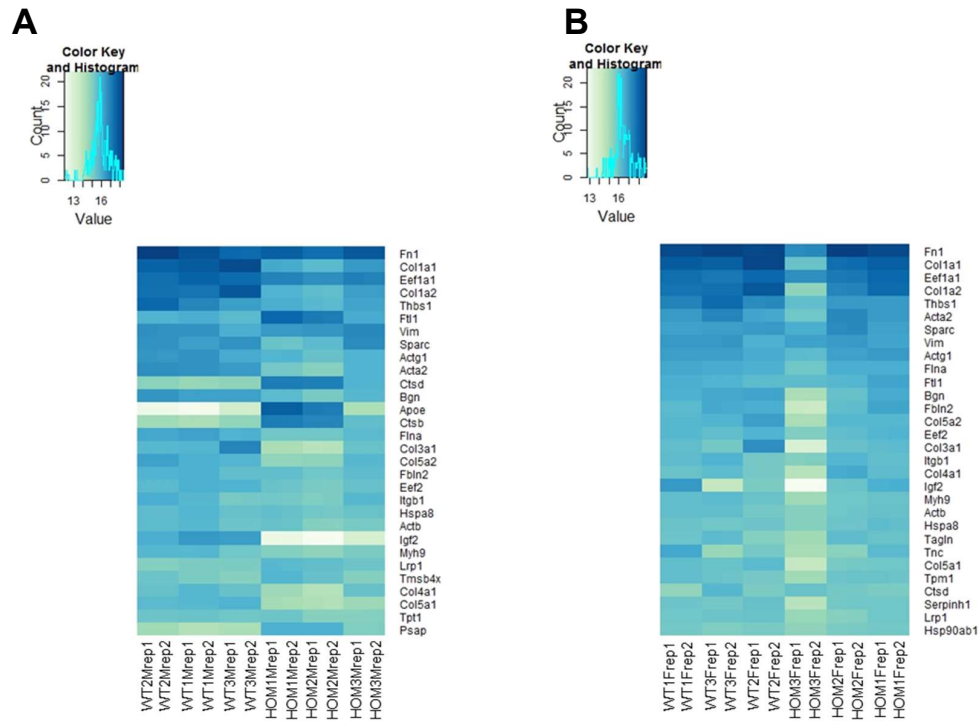


Figure 3.4 Heat maps demonstrating the expression level of the top 30 expressed genes in males (A) and females (B) HP1 γ WT and HOM MEFs. Variance-stabilising transformation was applied to obtain this data. The dark blue colour of the heat map represents high expression level while white colour represents low expression level. (A) Male HP1 γ WT and HOM MEFs showed high degree of similarity in expression level between technical replicates as well as biological replicates in most cases as indicated by the colour on the heat map. Dysregulation of the *Igf2* gene was revealed in which all of the male WT MEFs samples showed a higher expression level of this gene than HP1 γ HOM MEFs. Male WT samples and their technical replicates are: WT1Mrep1 and WT1Mrep2; WT2rep1 and WT2rep2; WT3rep1 and WT3rep2. Male HOM samples and their technical replicates are: HOM1Mrep1 and HOM1Mrep2; HOM2Mrep1 and HOM2Mrep2; HOM3Mrep1 and HOM3Mrep2. (B) Female HP1 γ WT and HOM MEFs showed high degree of similarity in expression level between technical replicates as well as biological replicates in most cases as indicated by the colour on the heat map. However, variation between biological replicates were observed in some genes including *Igf2* between biological replicates of both WT and HOM. Female WT samples and their technical replicates are: WT1Frep1 and WT1Frep2; WT2Frep1 and WT2Frep2; WT3Frep1 and WT3Frep2. Female HOM samples and their technical replicates are: HOM1Frep1 and HOM1Frep2; HOM2Frep1 and HOM2Frep2; HOM3Frep1 and HOM3Frep2.

3.1.3 HP1 γ regulates distinct subsets of genes in males and females

The different magnitude of effect revealed in male and female HP1 γ HOM MEFs prompted us to ask whether a common or distinct subset of genes were differentially expressed in male and female. For this, differentially expressed genes identified in male and female samples were compared and presented in a Venn diagram (Figure 3.5). As shown in Figure 3.5, 2258 genes and 623 genes were found to be upregulated only in male or female respectively. Similarly, there are 1569 genes and 97 genes which were downregulated only in male or female respectively. Two subsets of commonly regulated genes by both gender were also revealed. 378 genes and 71 genes are found up- or downregulated respectively in both male and female. Interestingly, two subsets of genes were found dysregulated in male and female in opposite directions in response to HP1 γ knockout. There are 9 genes upregulated in male and downregulated in female while 8 genes were downregulated in male and upregulated in female. These genes together with the fold change in response to HP1 γ deletion are listed in Table 3.1 and Table 3.2.

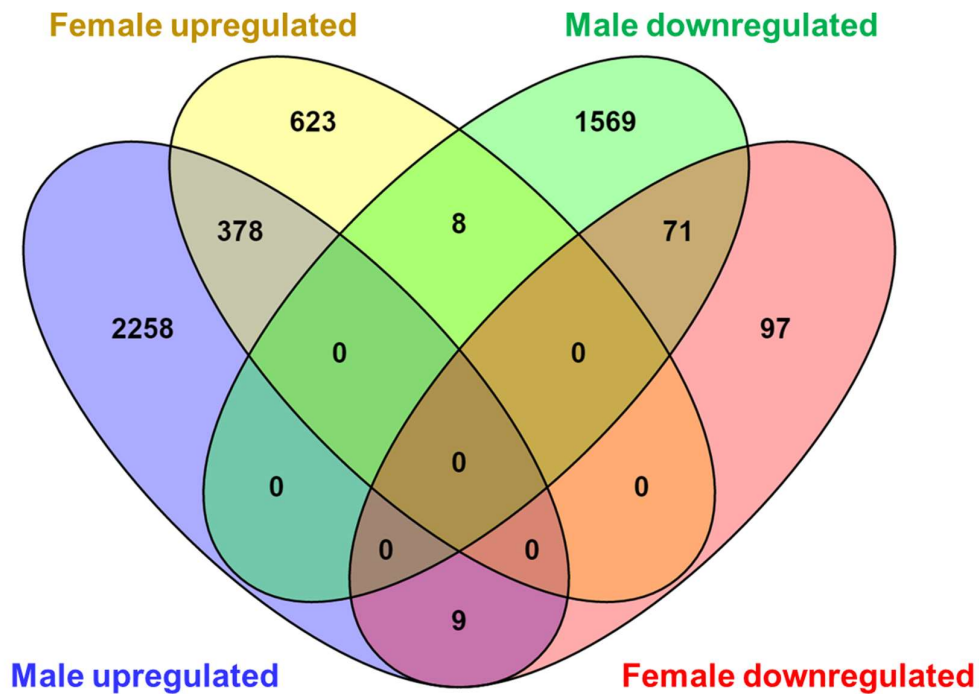


Figure 3.5 Venn diagram demonstrating the common or different subsets of genes differentially expressed in male and female HP1 γ HOM MEFs. Differentially expressed genes identified in male and female HP1 γ HOM MEFs relative to WT were compared. Common or distinct subsets of genes that are regulated by HP1 γ in male or female MEFs were revealed. Interestingly, genes that were differentially expressed in opposite directions in male and female were also revealed. Differentially expressed gene presented here are dysregulated for at least 1.5 fold with P-value<0.05. This figure is generated by Venny 2.0.2 (Oliveros, J.C. (2007-2015) Venny. An interactive tool for comparing lists with Venn's diagrams. (<http://bioinfogp.cnb.csic.es/tools/venny/index.htm>))

Genes UPregulated in male and DOWNregulated in female in response to HP1 γ knockout	Fold UPregulation in male relative to WT	Fold DOWNregulation in female relative to WT
<i>Gda</i>	6.68	2.07
<i>Ccl8</i>	19.16	2.00
<i>Spp1</i>	2.46	2.41
<i>Pgm2l1</i>	1.90	1.56
<i>Nxpe5</i>	2.39	2.79
<i>Fbxw10</i>	1.90	1.66
<i>Mmp13</i>	2.64	2.14
<i>Ppbp</i>	2.22	2.62
<i>Tgm2</i>	1.69	1.52

Table 3.1 List of identified genes which are upregulated in male and downregulated in female MEFs in response to HP1 γ deletion. A subset of genes were found dysregulated in male and female in opposite direction upon HP1 γ knockout in which 9 genes were upregulated in male but downregulated in female as shown in this table.

Genes DOWNregulated in male and UPregulated in female in response to HP1 γ knockout	Fold DOWNregulation in male relative to WT	Fold UPregulation in female relative to WT
<i>Mdk</i>	2.07	2.23
<i>Ppp2r2b</i>	1.95	2.79
<i>Adamtsl2</i>	2.62	2.14
<i>Hif3a</i>	4.29	2.38
<i>Slc2a4</i>	2.91	2.17
<i>Col26a1</i>	2.99	2.11
<i>Hnrnph3</i>	1.67	1.55
<i>Pappa2</i>	2.20	1.95

Table 3.2 List of identified genes which are downregulated in male and upregulated female MEFs in response to HP1 γ deletion. A subset of genes were found dysregulated in male and female in opposite direction upon HP1 γ knockout in which 8 genes were downregulated in male but upregulated in female as shown in this table.

3.1.4 Gene Ontology (GO) analysis implicates HP1 γ in important biological processes

In order to investigate the physiological functions influenced by the loss of HP1 γ , Gene Ontology (GO) analysis was employed to identify functional groupings of the differentially expressed genes. Due to the difference in magnitude of effect observed in males and females as shown in the previous section, this analysis was done separately on male and female samples.

As expected, a much higher number of functional groups were associated with dysregulated gene in males compared to females. GO analysis revealed a total of 486 and 539 functional groups associated with genes upregulated and downregulated upon HP1 γ deletion respectively in males (Table S1 and S2 in appendix). In contrast, there are only 5 and 70 functional groups associated with genes upregulated and downregulated respectively upon HP1 γ deletion in females (Table S3 and S4 in appendix). This result suggest that males have a higher dependency on HP1 γ in maintaining their normal functions.

3.1.4.1 GO analysis of the effect of HP1 γ deficiency in males

Among the affected biological processes (BP) associated with genes downregulated upon HP1 γ deletion in male, enrichment of certain functional groups were observed. These enriched functional groups include those related to cell cycle and RNA processing which is consistent with previously published studies: RNA processing (Saint-Andre, Batsche et al. 2011, Smallwood, Hon et al. 2012), cell cycle progression (Abe, Naruse et al. 2011) and chromatid segregation (Obuse, Iwasaki et al. 2004, Serrano, Rodriguez-Corsino et al. 2009, Shimura, Toyoda et al. 2011, Grzenda, Leonard et al. 2013) (Table S2 in appendix).

Relatively less pronounced enrichment of groups related to DNA damage response, regulation of gene expression, and some histone/chromatin related groups were also observed. Enrichment of functional groups in regulation of gene expression and histone/chromatin related processes is consistent with the role of HP1 γ being an

epigenetic modifier involved in regulation of gene expression (Reviewed in - Hiragami and Festenstein 2005, Kwon and Workman 2011). Moreover, that HP1 γ has been implicated in DNA damage repair has been previously suggested by a number of studies (Lee, Kuo et al. 2013, Soria and Almouzni 2013, Akaike, Kuwano et al. 2014, Wu, Nishikawa et al. 2015).

Interestingly, these functional groups are almost exclusively associated with genes downregulated upon loss of HP1 γ implying that the presence of HP1 γ is important for maintaining the expression level of these genes and hence their associated biological functions. The question arises as to how HP1 γ maintains transcriptional activity of these genes (see discussion section 4.1).

Within the group of genes upregulated upon HP1 γ deletion, there was enrichment of a large number of functional groups related to regulation of immune responses including immune-cell specific processes (Table S1 in appendix). Given that the cell model we used in this experiment is a fibroblast and not a specialised immune cell, this result suggests that HP1 γ might be involved in maintaining the cell type specific silencing of genes. For further analysis of this dataset addressing cell-type specificity see section 3.15.

3.1.4.2 GO analysis of the effect of HP1 γ deficiency in females

Enrichment of functional groups related to cell cycle were also found to be associated with downregulated genes in females (Table S4 in appendix). However, a smaller number of these groups were observed associated with female when compared to male. This difference in number suggests that regulation of cell cycle may also be affected in female MEFs in response to knockout of HP1 γ . However, the magnitude of the effect is likely to be smaller.

There are only 5 functional groups associated with upregulated gene in female in response to HP1 γ knockout and none of these groups were related to regulation of immune response which has been observed in male (Table S3 in appendix).

Collectively, these data suggest that HP1 γ is indeed playing diverse roles: positively regulating genes for essential cellular processes as well as negatively regulating genes for cell type specific functions. Also, this experiment implicates that HP1 γ in the regulation of important biological processes in MEFs. However, an unbiased analysis is required to test for the significance of this observation (see next section). Moreover, the higher number of affected biological processes revealed in males compared with females suggest that males have a higher dependency on HP1 γ for maintaining their normal gene expression profile.

3.1.5 Statistical overrepresentation test implicates HP1 γ in important biological processes

In order to have an unbiased way to confirm the observed enrichment of functional groups with the GO analysis presented in section 3.1.4, a statistical overrepresentation test (Mi, Muruganujan et al. 2013) was employed. In this analysis, dysregulated genes associated with males or females in response to HP1 γ knockout were compared to the mouse reference gene list and the fold enrichment of significantly over- or underrepresented GO biological processes were determined (Table S5 to S8 in appendix). The definition of the top 10 overrepresented biological processes can be found in appendix Table S9 to S12.

In general, genes dysregulated in males showed a higher number of significantly ($P < 0.05$) over- and underrepresented BP groups than females. A total of 41 and 39 groups were found to be associated with genes upregulated and downregulated upon HP1 γ deletion respectively in males (Table S5 and S6 in appendix). In contrast, there were only 22 and 9 functional groups associated with genes upregulated and downregulated respectively upon HP1 γ deletion in females (Table S7 and S8 in appendix).

3.1.5.1 Statistical overrepresentation test of the effect of HP1 γ deficiency in males

Consistent with the observation reported in the previous section (section 3.1.4), analysis of downregulated genes in HP1 γ HOM males revealed overrepresentation of groups related to cell cycle and RNA processing (Table S6 in appendix). Among the processes related to cell cycle, ‘regulation of cell cycle’ showed the highest enrichment of 6.94 fold. ‘Regulation of cell cycle’ is also the highest enriched group revealed among the whole list. The group ‘chromosome segregation’ also showed high enrichment of 4.12 fold. Other cell cycle related groups revealed include ‘mitosis’ (2.49 fold enriched) and ‘cell cycle’ (2.05 fold enriched). All of the revealed groups related to RNA processing showed a similar magnitude of overrepresentation. These groups include ‘mRNA splicing, via spliceosome’ (3.16

fold enriched), 'RNA splicing, via transesterification reactions' (2.82 fold enriched), 'RNA splicing' (2.76 fold enriched) and 'mRNA processing' (2.39 fold enriched). Moreover, 'DNA repair' which was observed in the GO analysis as presented in the previous section (section 3.1.4) was also found to be overrepresented with an enrichment of 3.95 fold.

Consistent with the observation reported in the previous section (section 3.1.4), enrichment of processes related to the immune response were found to be associated with the upregulated genes in males in response to HP1 γ knockout (Table S5 in appendix). These processes include some relatively general functions which can potentially be found in many cell types: 'response to interferon-gamma' (3.61 fold enriched), 'antigen processing and presentation' (3.37 fold enriched), 'Immune response' (2.08 fold enriched), and 'Immune system process' (1.92 fold enriched). Also, processes which are apparently specific to immune cells were revealed. These processes include the 'B cell mediated immunity' (2.42 fold enrichment) which was defined as "any process involved with the carrying out of an immune response by a B cell, through, for instance, the production of antibodies or cytokines, or antigen presentation to T cells" and 'macrophage activation' (2.28 fold enrichment) which was defined as "a change in morphology and behaviour of a macrophage resulting from exposure to a cytokine, chemokine, cellular ligand, or soluble factor". The genes contributing to these immune cell specific processes are shown in Table S13 and S14 in appendix. Indeed, some of these genes were specific to B cell or macrophage functions perhaps indicating that HP1 γ might be regulating tissue specific gene expression and preventing inappropriate expression in male MEFs.

3.1.5.2 Statistical overrepresentation test of the effect of HP1 γ deficiency in females

Unlike the male samples and the GO analysis presented in the previous section, the statistical overrepresentation test of the downregulated genes in female HP1 γ HOM MEFs did not reveal overrepresentation of groups related to cell cycle, RNA processing and DNA repair (Table S8 in appendix). Rather, most of the overrepresented groups revealed are related to developmental processes.

In contrast to the data obtained from male MEFs, none of the overrepresented BP groups associated with upregulated genes in female HP1 γ HOM MEFs are related to regulation of immune response (Table S7 in appendix). Instead, overrepresentation of some processes involved in cell adhesion were revealed. All of these groups showed similar levels of enrichment. ‘Cell-cell adhesion’ showed 3.51 fold enrichment while ‘cell adhesion’ and ‘biological adhesion’ showed 2.88 and 2.75 fold enrichment respectively.

Taken together, the results obtained from the statistical overrepresentation test are largely in line with the GO analysis. Data obtained suggest that HP1 γ is implicated in regulation of important biological processes in both male and female MEFs. In the analysis of male samples, HP1 γ was shown to play diverse roles: positively regulating genes for essential cellular processes (cell cycle, RNA processing and DNA damage response) as well as negatively regulating genes for cell type specific functions (B cell and macrophage mediated immunity). The higher number of over- and underrepresented biological processes revealed in males compared with females suggests that males have a higher dependency on HP1 γ for maintaining their normal gene expression profile. However, the level of dependency on HP1 γ for males and females may actually be process-specific in that the GO terms obtained from female samples were different from that of the males. These results therefore may reflect sexual dimorphism in both gene expression and response to HP1 deficiency. It is possible that HP1 is compensating for an underlying difference in gene expression between the sexes that is due to the different sex chromosome complement (XX versus XY).

3.1.6 HP1 γ is implicated in regulation of sexually dimorphic expression of genes

Observed differences between the sexes in response to HP1 γ knockout (Figure 3.3) prompted as to ask whether HP1 γ plays a role in regulating sexually dimorphic expression of genes. To address this question, I firstly identified the genes that are differentially expressed in HP1 γ WT male and female MEFs (Figure 3.6 and Figure 3.7). Thus, 176 genes were found to be differentially expressed by at least 1.5 fold (P-value <0.05) in HP1 γ WT male and female MEFs (Figure 3.6 and Figure 3.7, Table S15 and S16 in appendix). 114 genes had higher expression in females while 62 genes had higher expression in males (Figure 3.6 and Figure 3.7). Among these 176 sexually dimorphic genes, 10 of them are X- or Y-linked genes (Figure 3.7, yellow and red dots) while 166 genes are autosomal genes (Figure 3.7, blue dots). As expected, the Y chromosome genes were only expressed in males while the X chromosome genes were expressed in both sexes (Figure 3.7).

Next, the sensitivity of these 176 sexually dimorphic genes to HP1 γ deficiency was tested in both male and female. In response to HP1 γ knockout, 121 of the sexually dimorphic genes were dysregulated by at least 1.5 fold (P-value <0.05) in males (Figure 3.8A). Among these 121 dysregulated genes, 86 (71.1% of total number of dysregulated genes) of them were upregulated while 35 (28.9% of total number of dysregulated genes) of them were downregulated upon HP1 γ knockout (Figure 3.8A). The percentage of upregulated sexually dimorphic genes (86/176 i.e. 48.9%) was significantly enriched in this subset of genes compared to the percentage of the total number of upregulated genes (2645/23420 i.e. 11.3%) in males (Chi-square test: P<0.0001). The number of dysregulated genes was much lower in females where only 38 genes were significantly changed (Fold change >1.5 fold, P-value <0.05) (Figure 3.8B). 20 (~52.6% of total number of dysregulated genes) and 18 (47.4% of total number of dysregulated genes) genes were up- and downregulated respectively in female HP1 γ HOM MEFs (Figure 3.8B). In females, the percentage of upregulated sexual dimorphic genes (38/176 i.e. 21.6%) was also enriched compared to the percentage of the total number of upregulated genes (1009/23420 i.e. 4.3%) (Chi-square test: P<0.0001).

The higher number of upregulated than downregulated genes revealed in both males and females suggests that HP1 γ acts more often as a silencer to repress the expression of these sexually dimorphic genes. Also, the higher magnitude of effect (number of dysregulated genes) of HP1 γ on the regulation of sexually dimorphic genes in males suggests that males have a higher dependency on HP1 γ for maintaining the expression level of these sexual dimorphic genes which are set at a lower level in WT males than WT females.

In order to investigate whether HP1 γ is playing a role in maintaining the gender-specific expression of the sexually dimorphic genes, the genes that are expressed at a relatively higher level in males (62 genes) (Figure 3.10A) or females (114 genes) (Figure 3.9A) were tested for their sensitivity to HP1 γ deficiency separately in males and females. 82 genes out of the 114 genes that are expressed at a higher level in WT females were significantly changed upon HP1 γ knockout in males (Figure 3.9B). Strikingly, all of these genes were upregulated in the absence of HP1 γ (Figure 3.9B). This result suggests that in a WT condition, HP1 γ may prevent these genes from being much higher in males and hence maintain the difference between males and females. In female, most of these genes (91 genes out of 114 genes) were not significantly changed (Figure 3.9C). Among the 23 dysregulated genes, 7 and 16 genes were up- and downregulated in female HP1 γ HOM MEFs respectively (Figure 3.9C).

The 62 genes that are expressed at a higher level in WT males were also analysed (Figure 3.10A). In male, 38 of these genes were dysregulated significantly (Figure 3.10B). Most of these dysregulated genes were downregulated upon HP1 γ knockout (35 genes) while only 3 genes were upregulated (Figure 3.10B) indicating that in males most of these genes require HP1 γ to keep them at a higher level than that seen in females. In contrast, out of the 15 dysregulated genes in female, except 2 gene, all of these genes were upregulated in response to HP1 γ knockout (Figure 3.10C). This indicates that in females HP1 γ plays a predominantly repressive role on this group of genes. Overall, this result again suggests HP1 γ may be playing a role in maintaining the expression level of these sexual dimorphic genes in both males and females and hence to maintain the difference between the two genders.

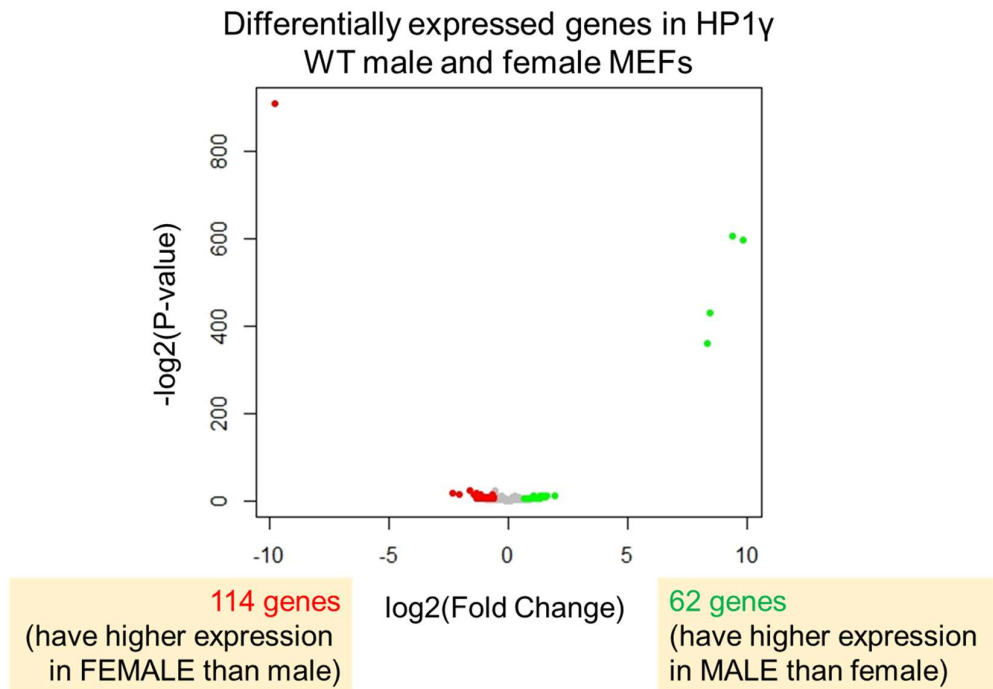


Figure 3.6 Volcano plot demonstrating the differentially expressed genes in HP1 γ WT male and female MEFs. Red and green dots represent genes that were differentially expressed by at least 1.5 fold. Grey dots represent genes that are not significantly changed. X axes of these plots represent the log₂ fold change of the differentially expressed genes while Y axes represent the $-\log_2$ P-value. Out of the total of 176 differentially expressed genes, 114 genes have higher expression in female while 62 genes have higher expression in male. Fold change >1.5, $P < 0.05$, $n=3$.

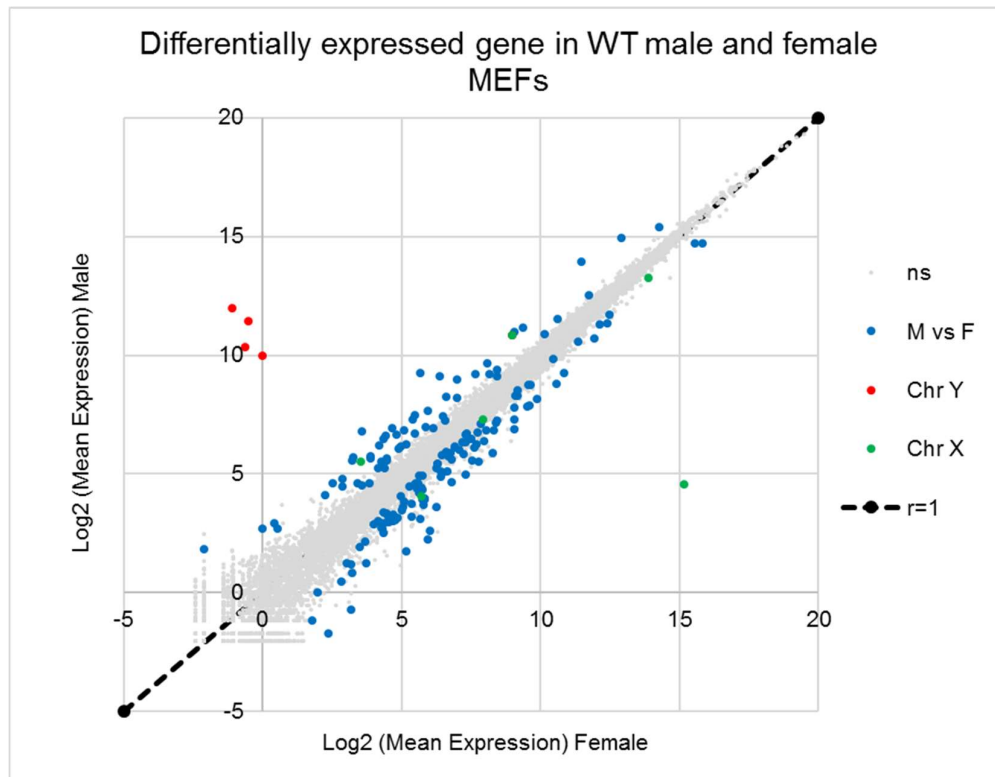


Figure 3.7 Density plot of differentially expressed genes comparing HP1 γ WT male and female MEFs. Blue dots represent autosomal genes that were differentially expressed by at least 1.5 fold when comparing males and females. Green and red dots represent the sexually dimorphic X- and Y-linked genes respectively. Grey dots represent genes that are not significantly changed. Dotted black line indicates fold change of 1. 176 genes were found to be differentially expressed in WT male and female MEFs by at least 1.5 fold. Among these 176 differentially expressed genes, 114 genes were expressed at a higher level in females (genes represented by the dots below the orange line) while 62 genes were expressed at a higher level in males (genes represented by the dots above the orange line). Fold change >1.5, $P < 0.05$, $n = 3$.

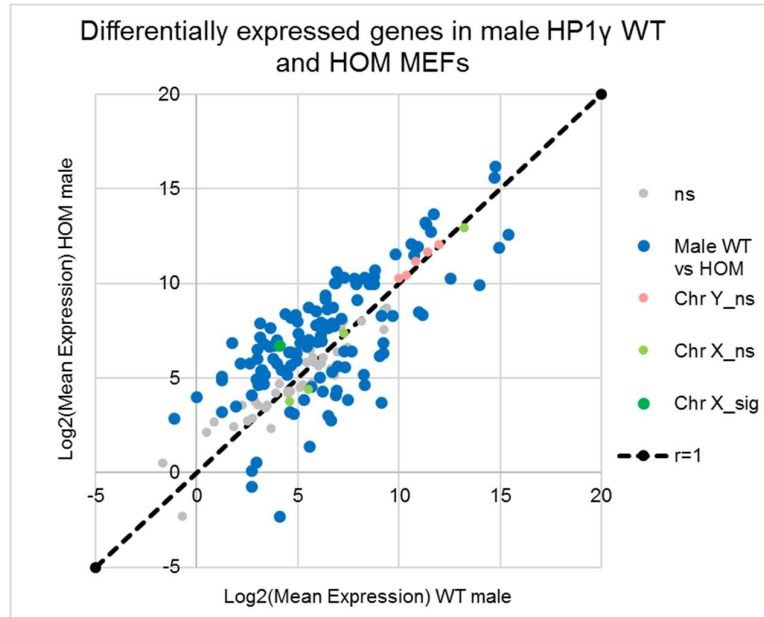
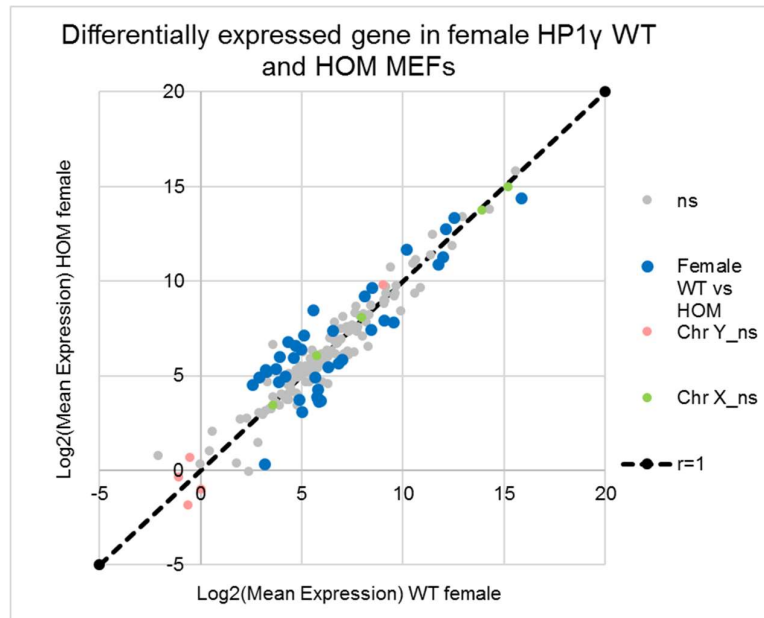
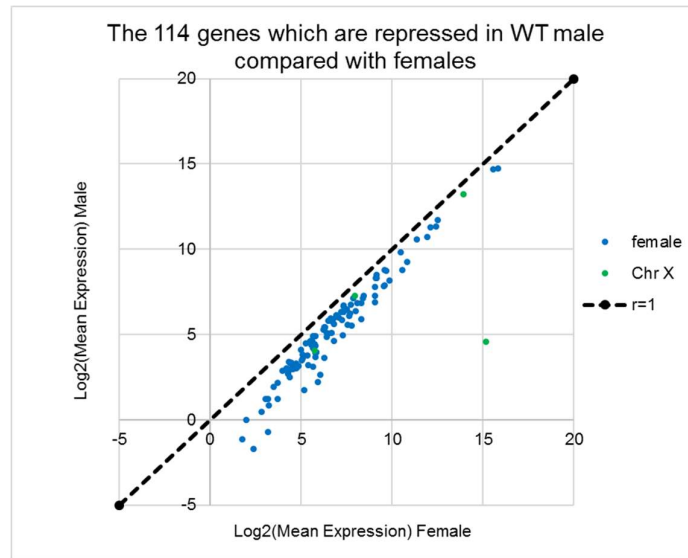
A**B**

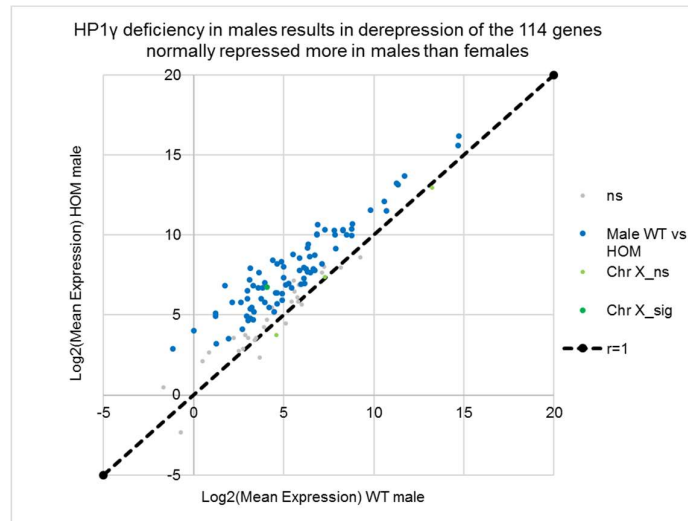
Figure 3.8 Density plots showing the effect of HP1 γ deficiency on the expression of the 176 sexually dimorphic genes identified in male and female MEFs. Blue dots represent autosomal genes that were differentially expressed by at least 1.5 fold when comparing WT and HP1 γ deficient MEFs (HOM). Pink dots represent the sexually dimorphic Y-linked genes that were not significantly changed. Light green dots represent the sexually dimorphic X-linked genes that were not significantly changed. Green dot represents the sexually dimorphic X-linked gene that was significantly changed upon HP1 γ knockout. Grey dots represent the sexually dimorphic genes that are not significantly changed upon HP1 γ knockout. Dotted black line indicates fold change of 1. (A) In response to HP1 γ knockout, 121 of the

sexual dimorphic genes were dysregulated by at least 1.5 fold in male. 86 genes were upregulated (genes represented by the dots above the black line) while 35 genes were downregulated (genes represented by the dots below the black line). (B) A relatively milder effect was observed in female in which only 38 genes were significantly changed by at least 1.5 fold. 20 genes and 18 genes were up- (genes represented by the dots above the black line) and downregulated (genes represented by the dots below the black line) respectively in female HP1 γ HOM MEFs. Fold change >1.5, $P < 0.05$, $n = 3$.

A



B



C

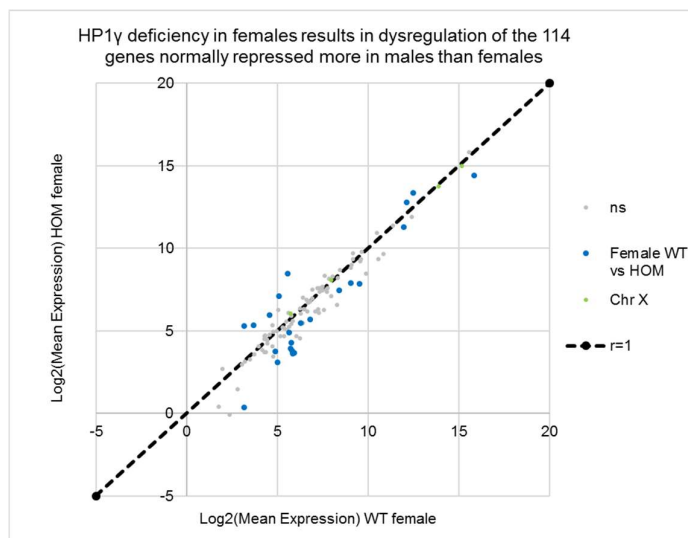
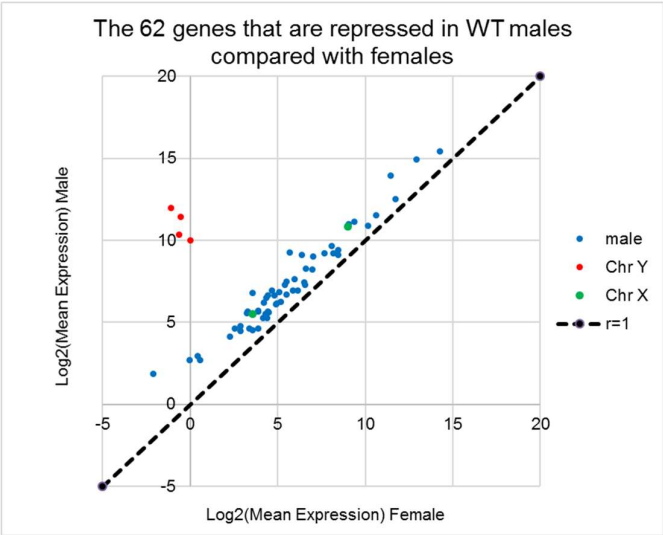
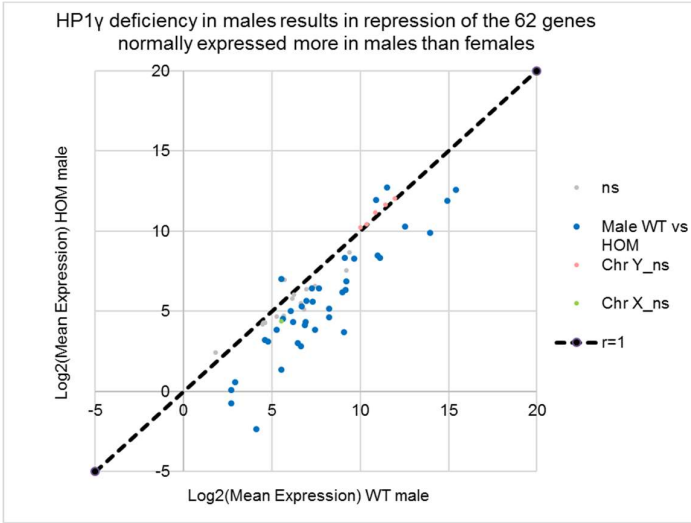


Figure 3.9 Density plots demonstrating the changes in the expression level of the 114 sexually dimorphic genes with higher expression level in WT female MEFs in response to HP1 γ knockout in both genders. Blue dots represent autosomal genes that were differentially expressed by at least 1.5 fold. Green and red dots represent the significantly changed sexually dimorphic X- and Y-linked genes respectively. Pink dots represent the sexually dimorphic Y-linked genes that were not significantly changed. Light green dots represent the sexually dimorphic X-linked genes that were not significantly changed. Grey dots represent genes that are not significantly changed. Dotted black line indicates fold change of 1. (A) There are 114 genes which are expressed at a higher level in WT female MEFs compared to WT male MEFs. (B) In male MEFs, 82 of these 114 sexual dimorphic genes were dysregulated in absence of HP1 γ . All of these 82 dysregulated genes were downregulated. (C) In female MEFs, 23 of these 114 sexual dimorphic genes were dysregulated in absence of HP1 γ . 7 and 16 genes were up- and downregulated upon HP1 γ knockout. Fold change >1.5, P<0.05, n=3.

A



B



C

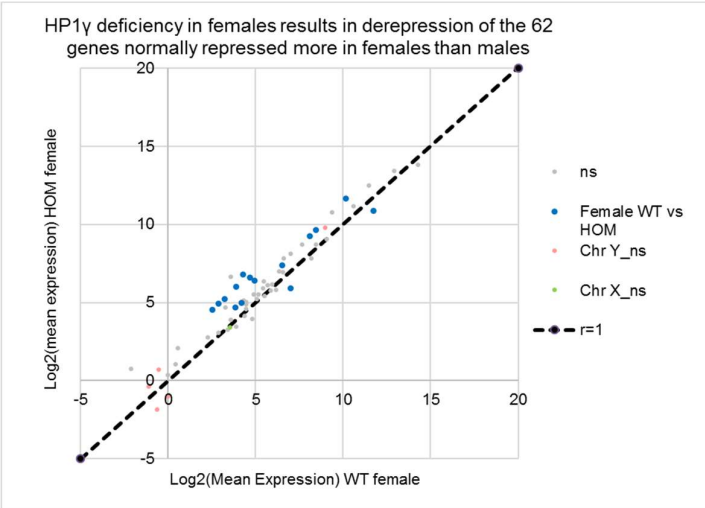


Figure 3.10 Density plots demonstrating the changes in the expression level of the 62 sexually dimorphic genes which have higher expression level in WT male MEFs in response to HP1 γ knockout in both genders. Blue dots represent autosomal genes that were differentially expressed by at least 1.5 fold. Green and red dots represent the significantly changed sexually dimorphic X- and Y-linked genes respectively. Pink dots represent the sexually dimorphic Y-linked genes that were not significantly changed. Light green dots represent the sexually dimorphic X-linked genes that were not significantly changed. Grey dots represent genes that are not significantly changed. Dotted black line indicates fold change of 1. (A) There are 62 genes which are expressed at a higher level in WT male MEFs compared to WT female MEFs. (B) In male MEFs, 38 of these 62 sexual dimorphic genes were dysregulated in absence of HP1 γ . 35 out of 38 dysregulated genes were downregulated. (C) In female MEFs, 15 of these 62 sexual dimorphic genes were dysregulated in absence of HP1 γ . 13 of these dysregulated genes were upregulated except 2 genes which was downregulated. Fold change >1.5, P<0.05, n=3.

3.1.7 HP1 γ is implicated in regulation of a subset of imprinted genes

As mentioned in section 3.1.2 of this chapter, dysregulation of the imprinted gene *Igf2* was revealed in male MEFs in response to HP1 γ knockout (Figure 3.4A). This finding prompted us to ask whether other imprinted genes were also affected.

Out of the 121 imprinted genes on our gene list, 13 genes showed no reads from the RNA-seq data obtained. 49 imprinted genes (~40% of total number of imprinted genes) were found to be dysregulated by at least 1.5 fold with an adjusted P-value <0.05 upon HP1 γ knockout (Figure 3.11). The percentage of dysregulated imprinted genes was significantly enriched compared to percentage of total dysregulated genes (~24%) of total number of gene in male (Chi-square test: P<0.0001). Among the 49 affected imprinted genes, 17 and 32 of these genes were up- and downregulated respectively (Figure 3.11) (Table S17 and S18 in appendix). This differs from the proportion of total genes affected (section 3.1.2) where more affected genes were upregulated, here more of the affected imprinted genes were downregulated. Interestingly, two of the most dramatically downregulated genes are the *Igf2* and *H19* genes which were downregulated by 12.9 and 13.3 fold respectively in response to HP1 γ knockout (Figure 3.12).

As mentioned in the introduction section 1.5, *Igf2* and *H19* genes are located in the same locus and are reciprocally expressed from both alleles according to the parental inheritance. In order to reveal whether imprinting is affected, methodology which is able to distinguish allelic specific expression is required. However, in this case RNA-seq cannot distinguish the allelic origin of the transcripts, and therefore we cannot determine whether genomic imprinting of the *Igf2/H19* cluster is affected in response to HP1 γ knockout.

However, if the imprint of the *Igf2/H19* cluster is dependent on HP1 γ , upon its removal, one might expect to see upregulation of *H19* and downregulation of *Igf2* genes (Figure 3.13B). Instead, dramatic downregulation to the same degree (approximately 13 fold) was observed for both *Igf2* and *H19* genes upon HP1 γ knockout. This result suggests that imprinting of this locus is unlikely to be affected. Rather, mechanisms which regulate expression of both genes may be affected.

Hence, we speculate that HP1 γ might be involved in regulating activity of the common enhancer of *Igf2* and *H19* genes (Figure 3.13C). However, this hypothesis has to be addressed by further experiments.

Fold change of imprinted genes upon HP1 γ knockout in male MEFs

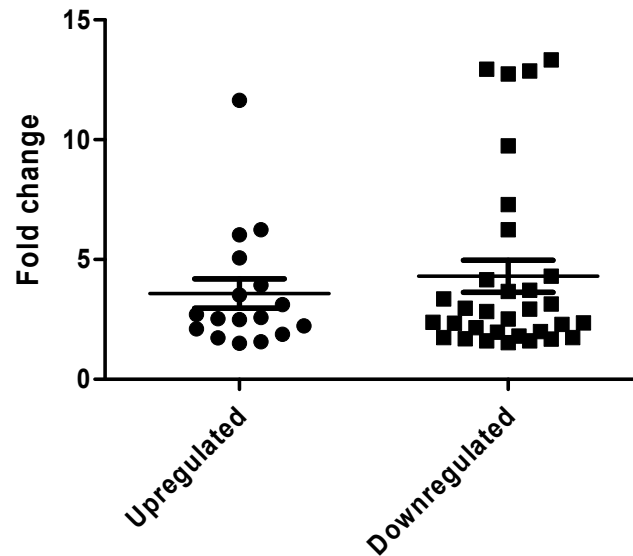
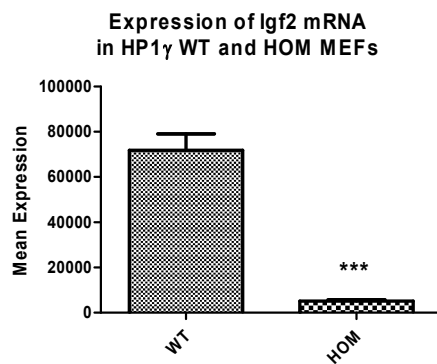


Figure 3.11 Fold change of imprinted genes upon HP1 γ knockout in male MEFs. Deficiency of HP1 γ in male MEFs led to dysregulation of 49 imprinted genes by at least 1.5 fold. 17 and 32 of these dysregulated genes were up- and downregulated respectively. Adjusted P-value <0.05, n=3.

A



B

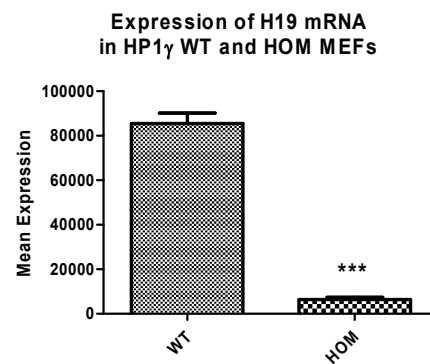


Figure 3.12 Expression of Igf2 (A) and H19 (B) mRNA in HP1 γ WT and HOM MEFs. RNA-seq revealed downregulation of Igf2 (A) and H19 (B) mRNA expression by 12.9 and 13.3 fold respectively in response to HP1 γ knockout. Mean expression represent the average of counts normalised by size factors (total number of reads). ***Adjusted P-value <0.0001, n=3.

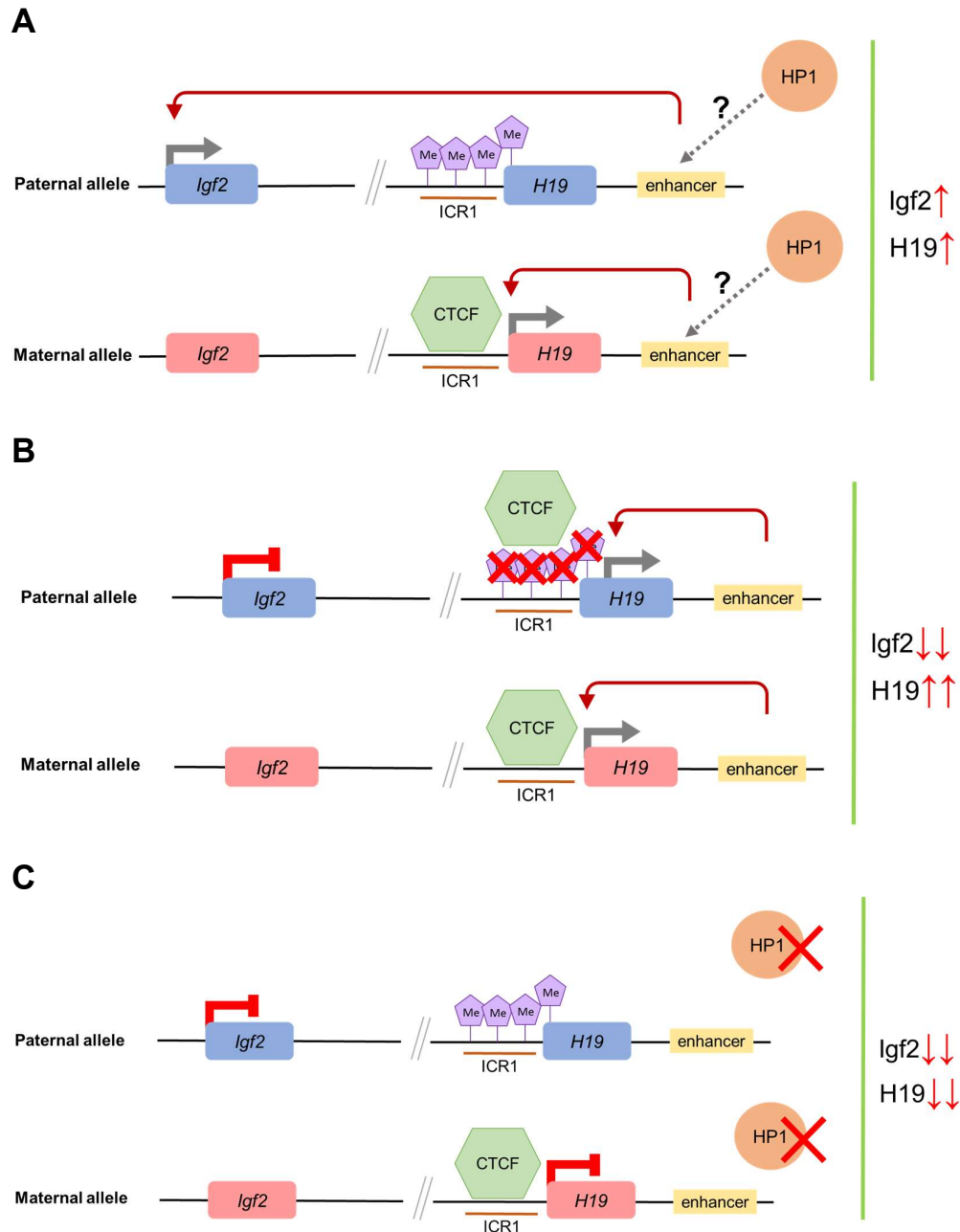


Figure 3.13 Schematic diagram demonstrating the putative model of regulation of the *Igf2/H19* gene cluster. (A) Model of the regulation of *Igf2/H19* gene cluster in the wild type situation. In wild type, methylation of the paternal ICR1 prevents binding of CTCF. The common enhancer can then act on the *Igf2* gene and allow its paternal expression. Binding of CTCF to ICR1 on the unmethylated maternal allele prevents the common enhancer from acting on the *Igf2* gene. Maternal *H19* is hence expressed. Our data showed that removal of HP1 γ caused dramatic downregulation of both *Igf2* and *H19* genes to the same degree. This result suggests that HP1 γ might be involved in regulating activity of the common enhancer of *Igf2* and *H19* genes by an unknown mechanism (see also Figure 3.13C). (B) Putative model of the regulation of *Igf2/H19* gene cluster if the maternal imprint has failed.

In this case, diminished DNA methylation on the ICR1 of the paternal allele allows binding of CTCF. CTCF on the paternal allele would block the enhancers from acting on the *Igf2* gene. In this situation, both paternal and maternal *H19* would be expressed causing overall upregulation of the *H19* gene. On the other hand, both paternal and maternal *Igf2* are repressed causing overall downregulation of the *Igf2* gene. (C) Putative model for the regulation of *Igf2/H19* gene cluster if enhancer function failed. In the case where enhancer mediated activation is compromised, both paternal *Igf2* and maternal *H19* which are both active in the wild type situation, would be repressed. Hence, overall expression of both *H19* and *Igf2* gene would be downregulated.

3.1.8 Summary of result section 3.1

This section focused on the role of HP1 γ in the regulation of gene expression. The main findings were as follows:

1. HP1 γ deficient MEFs were created and shown to have no HP1 γ expression providing a model system for studying the role of HP1 γ *ex vivo*.
2. Transcriptomic analysis shows that HP1 γ plays a role in both activating and repressing gene expression in MEFs
3. HP1 γ regulates distinct subsets of genes in males and females
4. The transcriptome of samples are more consistent amongst males within the same genotype group than females
5. The higher number of differentially expressed genes revealed in male MEFs compared to female MEFs suggests that males have a higher dependency on HP1 γ in maintaining their gene expression profile.
6. HP1 γ is implicated in regulating important biological processes and silencing of tissue specific genes. Difference in the number of biological processes in MEFs from the two genders suggests that males have a higher dependency on HP1 γ for maintaining their normal function.
7. HP1 γ is implicated in regulation of sexually dimorphic expression of genes
8. HP1 γ is implicated in regulation of a subset of imprinted genes. In the case of the *Igf2/H19* imprinted cluster, HP1 γ might be involved in regulation of the common enhancer and is required for high-level expression of both genes.

3.2 Investigation into the role of HP1 γ in the regulation of heterochromatin

3.2.1 Loss of HP1 γ causes derepression of mouse major satellite transcripts

The pericentromere of the mouse chromosome is a distinct chromatin structure that is adjacent to the centromere in the long arm of the chromosome (Reviewed in - Mouse Genome Sequencing, Waterston et al. 2002, Hall, Mitchell et al. 2012). It is composed predominately of the highly repetitive major satellite (Mj-sat) element (Joseph, Mitchell et al. 1989) and characteristic features consist of DNA methylation (Lewis, Meehan et al. 1992) and histone modifications H3K9me3 and H4K20me3 (Peters, O'Carroll et al. 2001, Lehnertz, Ueda et al. 2003, Schotta, Lachner et al. 2004).

Transcripts derived from the pericentromere, the so-called pericentric transcripts, were detected in various organisms including *S. pombe*, mouse and human (Reviewed in - Eymery, Callanan et al. 2009). In mammals, pericentric transcripts are normally expressed at low level (Eymery, Horard et al. 2009) and their expression has been considered to be a characteristic of instability and loss of integrity of heterochromatin (Eymery, Horard et al. 2009, Wang, Zhou et al. 2013) and thus a likely prerequisite for oncogenesis (Eymery, Callanan et al. 2009, Eymery, Horard et al. 2009). In fact, upregulation of these transcripts has not only been observed in some of the human cancers (Eymery, Horard et al. 2009, Ting, Lipson et al. 2011) but also a variety of processes including stress response, development, cell differentiation and potentially heterochromatin formation in mammals (Reviewed in - Eymery, Callanan et al. 2009). Although it is still unclear as to how the expression of these transcripts in mammals is regulated, the observed upregulation of these transcripts with these various processes indicates the importance in controlling the expression of these transcripts for maintenance of genome stability.

In the previous section (result section 3.1.2), transcriptomic analysis showed that HP1 γ is involved in both activation and repression of euchromatic genes. In order to elucidate its function in pericentric heterochromatin, HP1 γ WT and HOM MEFs

were examined for their Mj-sat expression by qRT-PCR. Deficiency of HP1 γ in MEFs led to an average of 5 fold (range from 3.1 to 37.6 fold) upregulation of Mj-sat relative to WT (Figure 3.14A). In view of the difference in magnitude of effect previously observed following transcriptomic analysis of male and female MEFs (see result section 3.1.2), this data was also analysed for the two genders individually. Figure 3.14B showed that there is no significant difference between the expression level of Mj-sat in male and female WT MEFs (Figure 3.14B). In response to HP1 γ knockout, both sexes showed similar levels of upregulation of Mj-sat expression in which the male and female MEFs have an average of 4.8 fold (range from 6.8 to 45.3) and 5.1 fold (range from 3.1 to 37.6 fold) upregulation relative to WT respectively (Figure 3.14C and D). Moreover, analogous effects have been observed in a human cell line. In HeLa cells, short hairpin RNA (shRNA)-mediated knockdown of HP1 γ led to an average of 5.5 and 14.4 fold upregulation of the human pericentric repeat elements α -satellite and γ -satellite variant II (γ -satellite II) expression relative to scramble control (Scr) respectively (Figure 3.15). Taken together, these results suggested that HP1 γ is a regulatory factor maintaining heterochromatin silencing. This effect is not gender-specific in mice and potentially conserved in human.

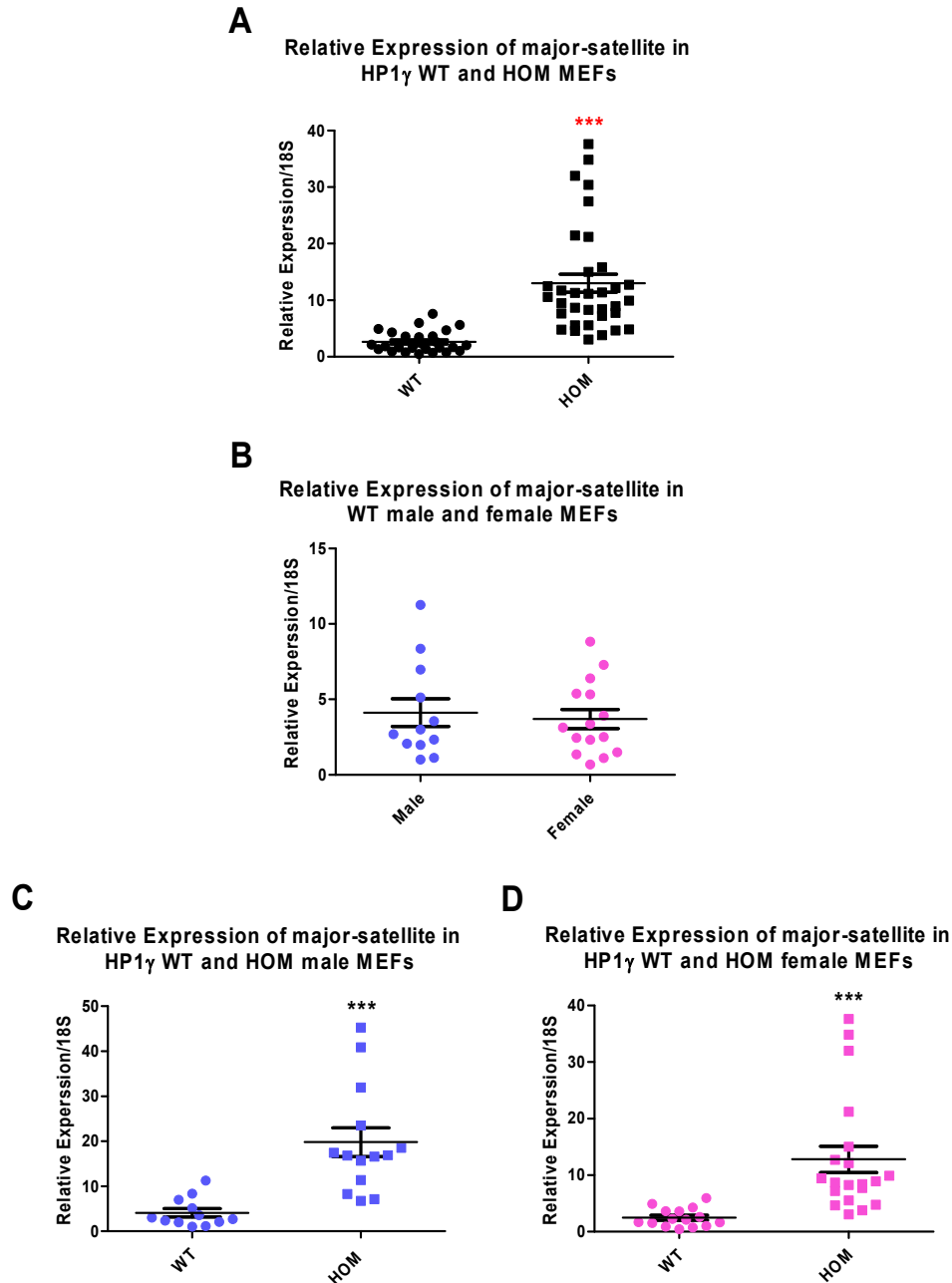


Figure 3.14 Relative expression of major satellite in HP1 γ WT and HOM MEFs. (A) Deficiency of HP1 γ in MEFs led to an average of 5 fold upregulation of major satellite (Mj-sat) in HP1 γ HOM MEFs relative to WT. (B) Male and female HP1 γ WT MEFs showed similar level of Mj-sat expression. (C and D) Both male and female MEFs showed approximately 5 fold upregulation of Mj-sat expression in absence of HP1 γ . Student's t-test: ***P<0.001 (compared to WT after being normalised against 18S expression). Error bars: SEM. n $\geq$ 27 for (A); n $\geq$ 12 for (B and C); n $\geq$ 15 for (D).

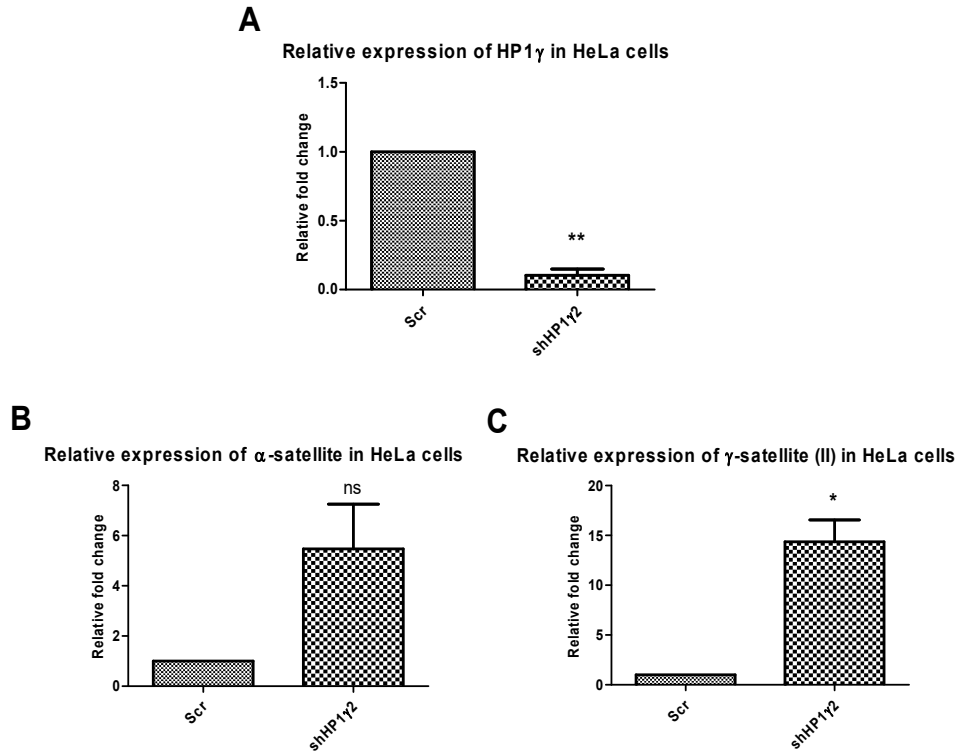


Figure 3.15 Relative expression of α -satellite and γ -satellite (II) in HeLa cells upon shRNA-mediated HP1 γ knockdown. (A) HP1 γ mRNA levels were significantly reduced in HeLa upon shRNA-mediated knockdown by HP1 γ -targeting lentivirus (shHP1 γ 2) to 10.3% of that of the non-targeting control with scrambled shRNA sequence (Scr). (B and C) Upon knockdown of HP1 γ , α -satellite and γ -satellite (II) were upregulated by an average of 5.5 and 14.4 fold respectively. Student's t-test: **P<0.005; *P<0.05; ns = not significant (relative to scramble control (Scr) after being normalised against β -actin expression). Error bars: SEM. n=3.

3.2.2 Loss of HP1 γ does not affect H3K9me3 and HP1 α nuclear distribution but HP1 β is redistributed in passage 1 nuclei

The upregulation of Mj-sat expression in HP1 γ HOM MEFs prompted us to ask whether the epigenetic signature of pericentric heterochromatin is disrupted in these cells. As mentioned previously (section 3.2.1), heterochromatin is characterised by its highly condensed structure and enrichment of the histone mark H3K9me3 which provides a binding site for the HP1 proteins (Bannister, Zegerman et al. 2001, Lachner, O'Carroll et al. 2001). Thus, the integrity of heterochromatin was examined using immunofluorescence microscopy to look at the staining pattern for 4',6-diamidino-2-phenylindole (DAPI) (for staining of DNA dense region), and deposition of the silencing histone mark H3K9me3 as well as heterochromatic HP1 isoforms HP1 α and HP1 β in HP1 γ WT and HOM MEF cells.

Firstly, the knockout phenotype of HP1 γ HOM MEFs was confirmed by immunofluorescence staining with HP1 γ -targeting antibody in which no detectable signal of HP1 γ was obtained in these cells (Figure 3.16C and D, right panels). This also acted as a negative control for HP1 γ antibody demonstrating that it is highly specific. In response to HP1 γ depletion, the staining pattern of DAPI did not show significant difference (Figure 3.16A to D, left panels) indicating condensed heterochromatin is still present in these cells. Consistent with previous studies, HP1 γ was detected in both euchromatin and heterochromatin compartments (Figure 3.16 A and B, right panels) (Minc, Allory et al. 1999, Nielsen, Ortiz et al. 1999, Kourmouli, Theodoropoulos et al. 2000, Minc, Courvalin et al. 2000, Nielsen, Oulad-Abdelghani et al. 2001). Two different staining patterns for HP1 γ were observed in HP1 γ WT MEFs: pan-nuclear distribution (81.9%) (Figure 3.16A) and its concentration within DAPI-dense regions (18.1%) (Figure 3.16B). These different distribution patterns might reflect different stages of the cell cycle (Minc, Allory et al. 1999).

As expected, staining of H3K9me3 of WT MEFs showed clear and distinct co-localisation with DAPI-dense regions (Figure 3.17A, right panel). However, no significant differences were observed with the depletion of HP1 γ (Figure 3.17B, right panel). The level of H3K9me3 on pericentric heterochromatin was also

examined by chromatin immunoprecipitation (ChIP) in WT and HP1 γ HOM MEFs. Figure 3.20A shows that the HP1 γ level on Mj-sat was dramatically reduced in HP1 γ HOM MEFs compared to WT. In response to the reduction of HP1 γ , no significant change was observed in H3K9me3 enrichment on Mj-sat as determined by ChIP (Figure 3.20B). These results suggest that large scale loss of H3K9me3 is unlikely to be responsible for the derepression of heterochromatin transcripts observed in HP1 γ HOM MEF. It is also consistent with a recent study showing that HP1 proteins are dispensable for the establishment of SUV39H-mediated H3K9me3 accumulation at the pericentromeric region (Muramatsu, Singh et al. 2013).

The nuclear localisation pattern of the other two HP1 isoforms (HP1 α and HP1 β) was also tested in the presence and absence of HP1 γ . Consistent with previous studies, about 97% and 79% of the nuclei stained with anti-HP1 α (Figure 3.18A, right panel) and anti-HP1 β (Figure 3.19A, right panel) antibody respectively showed distinct, but not exclusive association with heterochromatic regions as revealed by DAPI staining in WT MEFs (Minc, Allory et al. 1999, Nielsen, Ortiz et al. 1999, Kourmouli, Theodoropoulos et al. 2000, Minc, Courvalin et al. 2000, Nielsen, Oulad-Abdelghani et al. 2001). Both HP1 α and HP1 β were also found diffusely staining the nucleus in a small proportion of cells (3% for HP1 α (Figure 3.18B, right panel) and 21% for HP1 β (Figure 3.19B, right panel)). In contrast, immunofluorescent labeling of HP1 γ revealed that the majority of nuclei showed a diffuse pan-nuclear pattern (82%) (Figure 3.16A), while a small proportion of nuclei (18%) showed co-localisation of HP1 γ with DAPI-dense regions (Figure 3.16B). In response to deficiency of HP1 γ , no dramatic changes were observed in the distribution pattern of HP1 α indicating that recruitment of HP1 α to heterochromatic regions is independent of HP1 γ (Figure 3.18C, right panel). Strikingly, the proportion of cells that showed pan-nuclear distribution of HP1 β increased from 21.2% in the wild type to 74% in HP1 γ HOM MEFs (Figure 3.19A and C, right panel). As previous analysis of MEFs from these mice showed no disruption in the cell cycle this shift in distribution of patterns of staining are unlikely to reflect skewing of the cell cycle. It is possible that following removal of HP1 γ which is known to target euchromatic regions, HP1 β is now redistributed to these sites (see discussion section 4.5). Expression levels of HP1 α and HP1 β mRNA

were then examined by qRT-PCR. Figure 3.21 shows that both HP1 γ WT and HOM MEFs had similar levels of HP1 α and HP1 β mRNA. This data indicates that the different staining pattern of HP1 β in HP1 γ HOM MEFs is unlikely to be due to changes in the expression level of this protein. Table 3.3 (page 152) summarises the number of nuclei in passage 1 with different staining patterns scored and their percentage of total.

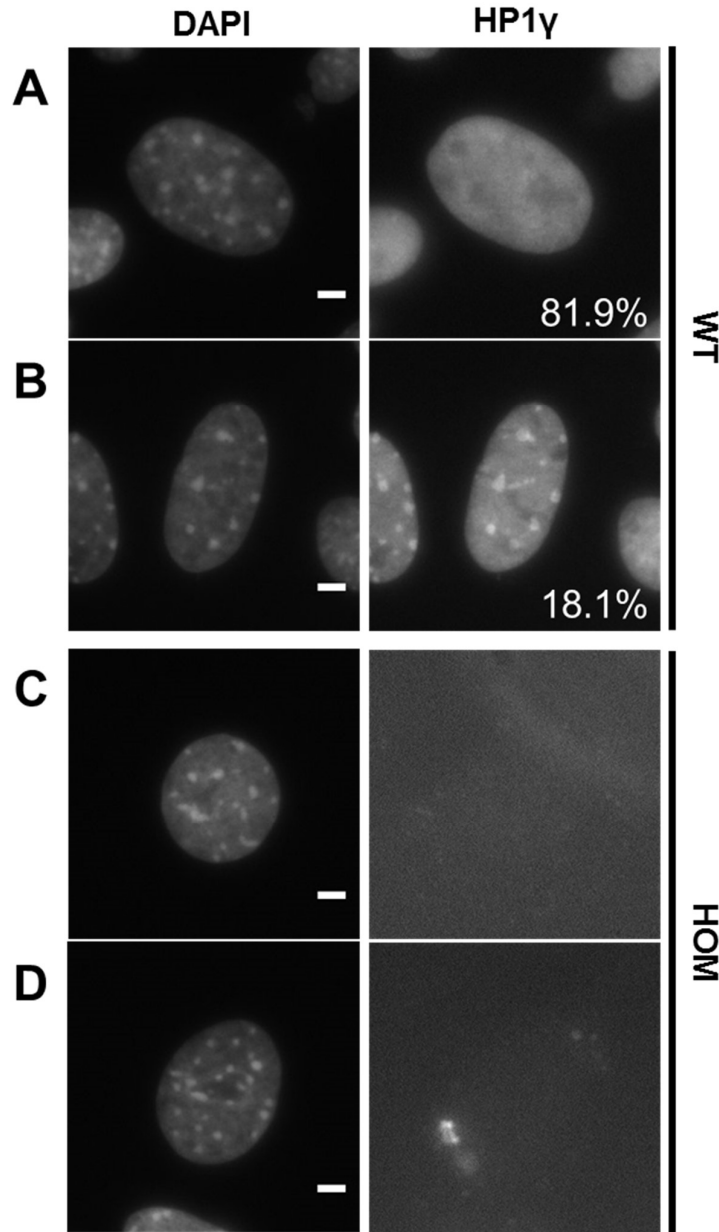


Figure 3.16 Immunofluorescence analysis of HP1 γ in HP1 γ WT and HOM MEFs. Representative images of nuclei of different genotype are shown. (A and B) HP1 γ WT MEFs stained with anti-HP1 γ antibody displayed two different patterns of HP1 γ : pan-nuclear distribution and the preferential distribution concentrated within DAPI-dense region. (A to D) No significant difference in DAPI pattern was observed in HP1 γ WT and HOM MEFs. (C and D) No detectable signal of HP1 γ was obtained in HP1 γ HOM MEFs demonstrating the high specificity of antibody. Percentages of counted nuclei that displayed the described phenotype are indicated by white text at the bottom right corners of the images. Scale bar, 5 μ m. These images were generated by Leica widefield fluorescence microscope and analysed with ImageJ.

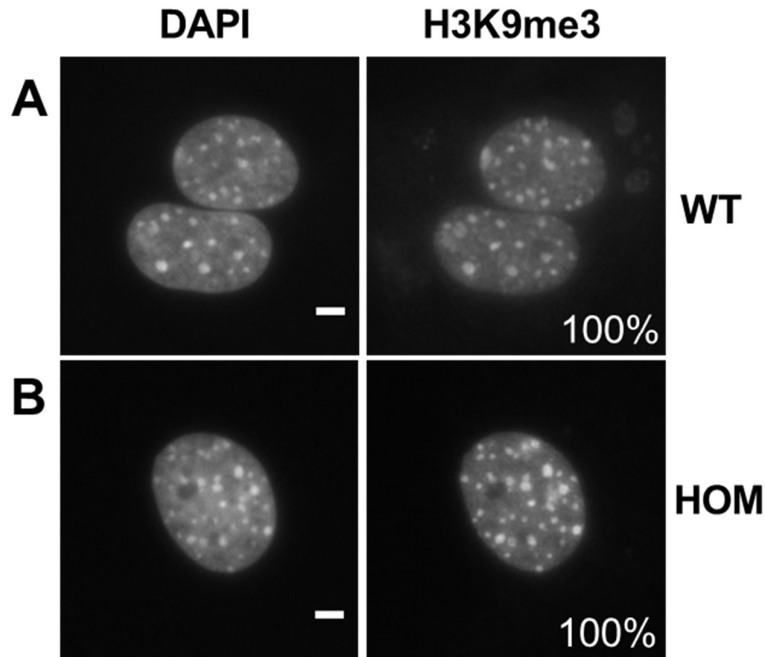


Figure 3.17 Immunofluorescence analysis of H3K9me3 in HP1 γ WT and HOM MEFs. Representative images of nuclei of different genotype are shown. (A) WT MEFs showed clear and distinct co-localisation of H3K9me3 and DAPI signals. (B) No significant difference between H3K9me3 staining pattern in HP1 γ WT and HOM MEFs were observed. Percentages of counted nuclei that displayed the described phenotype are indicated by white text at the bottom right corners of the images. Scale bar, 5 μ m. These images were generated by Leica widefield fluorescence microscope and analysed with ImageJ.

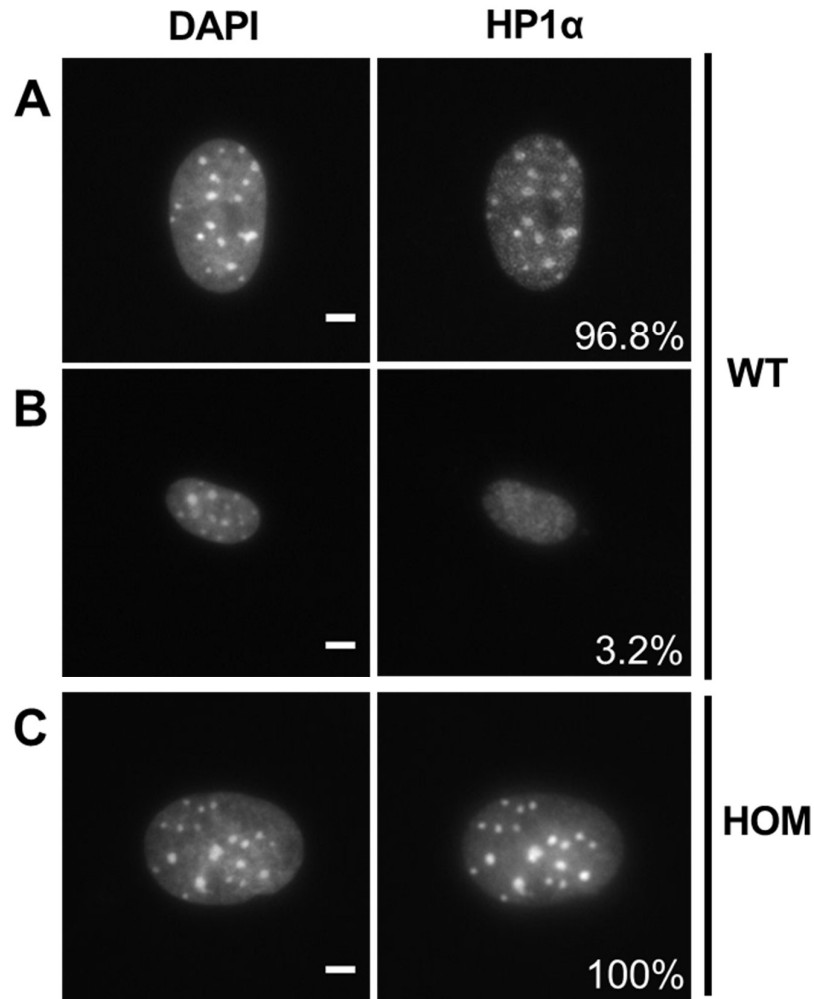


Figure 3.18 Immunofluorescence analysis of HP1 α in HP1 γ WT and HOM MEFs. Representative images of nuclei of different genotype are shown. (A and B) Majority of nuclei stained with anti-HP1 α antibody showed distinct but not exclusive association with DAPI dense regions in WT MEFs. (C) No dramatic changes were observed in the distribution pattern of HP1 α in response to deletion of HP1 γ . Percentages of counted nuclei that displayed the described phenotype are indicated by white text at the bottom right corners of the images. Scale bar, 5 μ m. These images were generated by Leica widefield fluorescence microscope and analysed with ImageJ.

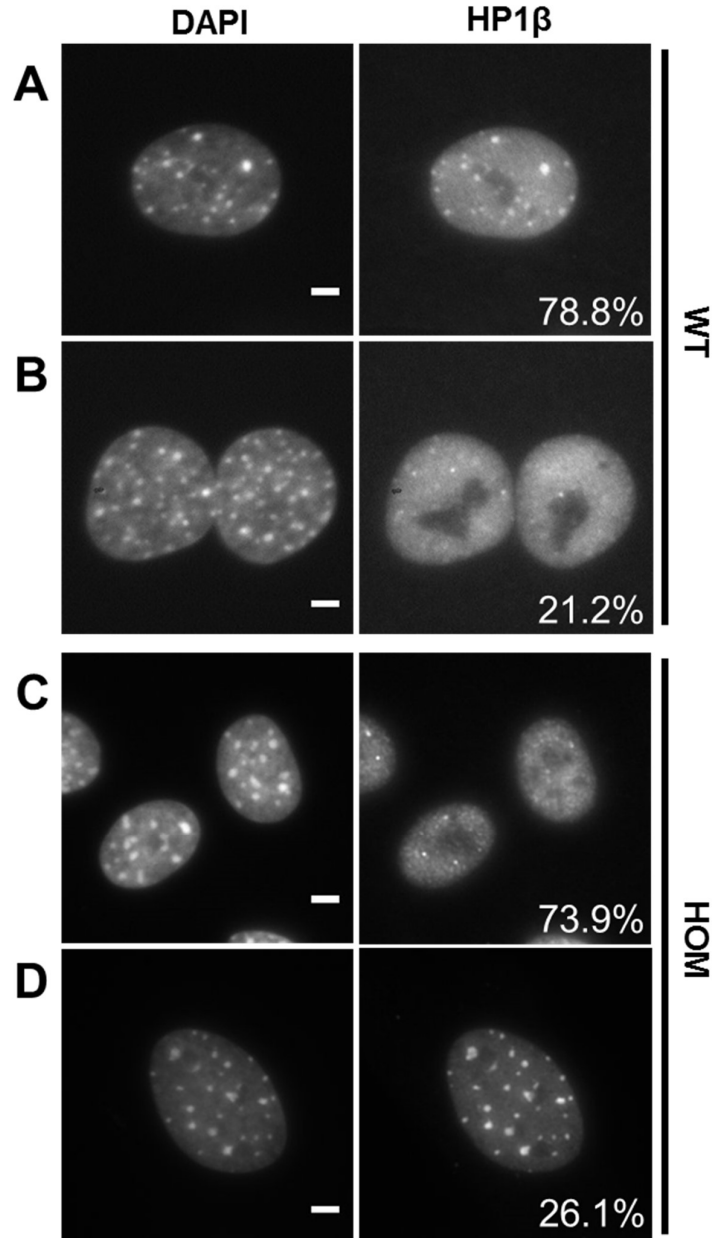


Figure 3.19 Immunofluorescence analysis of HP1 β in HP1 γ WT and HOM MEFs. Representative images of nuclei of different genotype are shown. (A and B) Most (78.8%) of nuclei stained with anti-HP1 β antibody showed distinct but not exclusive association with DAPI dense regions in WT MEFs while relatively small proportion of nuclei (21.2%) showed diffuse staining. (C and D) Staining pattern of HP1 β was changed in response to deletion of HP1 γ in which most (73.9%) of the nuclei showed diffuse staining of HP1 β . Percentages of counted nuclei that displayed the described phenotype are indicated by white text at the bottom right corners of the images. Scale bar, 5 μ m. These images were generated by Leica widefield fluorescence microscope and analysed with ImageJ.

Antibody	Genotype	Preferential distribution to DAPI-dense regions	Pan-nuclear distribution	Total number of nuclei scored
HP1 α	WT	96.8% (399)	3.2% (13)	412
	HOM	100% (393)	0% (0)	393
HP1 β	WT	78.8% (330)	21.2% (89)	419
	HOM	26.1% (131)	73.9% (370)	501
HP1 γ	WT	18.1% (154)	81.9% (697)	851
	HOM	N/A	N/A	N/A
H3K9me3	WT	100% (525)	0% (0)	525
	HOM	100% (440)	0% (0)	440

Table 3.3. A summary of quantitative analysis on the number of HP1 γ WT and HOM MEFs nuclei with differential staining patterns revealed by immunofluorescence. Number of nuclei with different staining patterns were scored as shown in brackets. These numbers were then converted into percentages of the total number of cells scored. N/A, not applicable. Chi-square test: P=0.0003 for HP1 α ; P<0.0001 for HP1 β data.

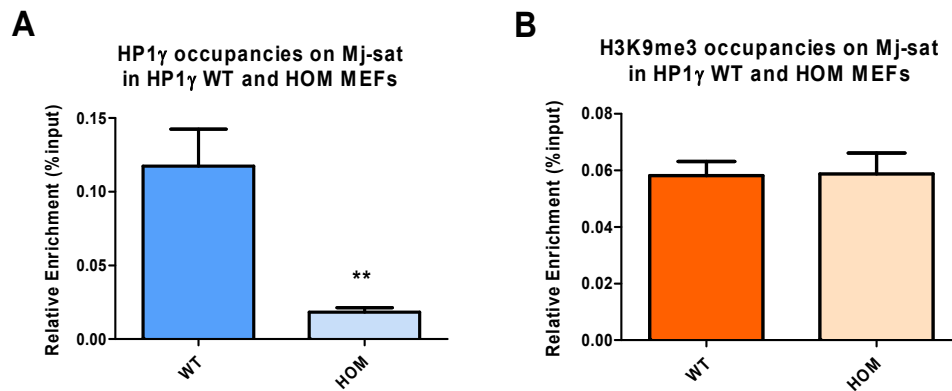


Figure 3.20 Relative enrichment of HP1 γ and H3K9me3 on major satellite in HP1 γ WT and HOM MEFs by ChIP. (A) HP1 γ HOM MEFs showed 15.6% signal of HP1 γ relative to WT which can be considered as the background given the absence of HP1 γ in these cells. (B) Relative enrichment of H3K9me3 on Mj-sat did not show significant difference between HP1 γ WT and HOM MEFs. Student's t-test: **P<0.01 (compared to WT after being normalised to input signal). Error bars: SEM. n $\geq$ 10 for (A); n $\geq$ 13 for (B).

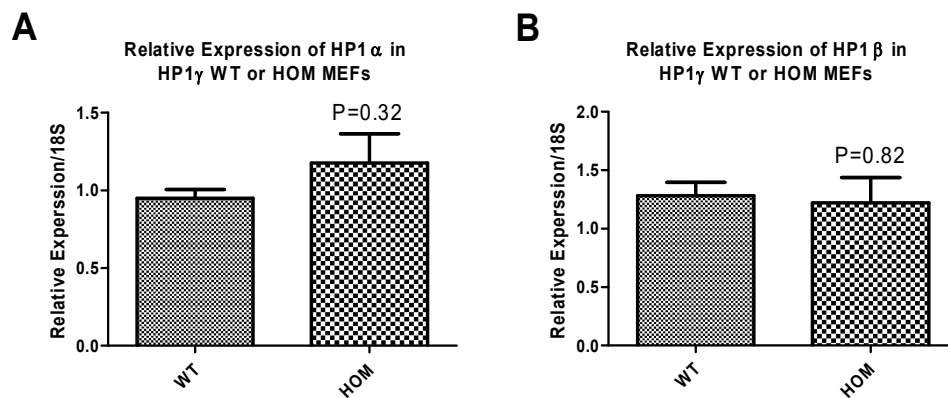


Figure 3.21 Relative expression of HP1 α and HP1 β mRNA in HP1 γ WT and HOM MEFs. (A and B) Both HP1 γ WT and HOM MEFs showed similar level of HP1 α and HP1 β mRNA. Error bars: SEM. $n \geq 5$. P-values were stated on the graph (compared to WT after being normalised against 18S expression).

3.2.3 Summary for result section 3.2

This section focused on the role of HP1 γ in maintaining transcriptional silencing at constitutive heterochromatin which is mainly composed of the Mj-sat repeat. The main findings were as follows:

1. HP1 γ is a regulatory factor which maintains silencing of Mj-sat transcripts in MEFs from both male and female mice and its role is potentially conserved in human.
2. Consistent to previous studies, HP1 γ was detected in both euchromatin and heterochromatin compartments but showed two different staining patterns under immunofluorescence microscopy: pan-nuclear distribution and the concentration within DAPI-dense regions.
3. Knockout of HP1 γ did not lead to changes in the level of H3K9me3 on the Mj-sat repeats element indicating that loss of H3K9me3 is either a) unlikely to be responsible for the derepression of Mj-sat transcripts observed in HP1 γ depleted MEFs or b) the derepression only occurs on a small subset of Mj-sat repeats and therefore the reduction in H3K9me3 might not have been detected.
4. Knockout of HP1 γ led to an increased proportion of cells with diffuse pan-nuclear pattern of HP1 β compared to WT.

3.3 Investigation into the context-dependent gene regulation of HP1 γ *in vivo*

In the section 3.1.2, RNA-seq data showing a large subset of genes up- or downregulated in male MEFs upon removal of HP1 γ were presented. This finding is consistent with various previous studies (see introduction section 1.4.2), implicating HP1 γ in regulation of both gene activation and repression. Affected physiological functions upon deletion of HP1 γ were also revealed by GO term analysis (see section 3.1.4 and 3.1.5) indicating the importance of the presence of HP1 γ in maintaining the normal function of the cell.

This interesting feature of HP1 γ being able to regulate both gene activation and repression in the genome prompted us to ask how the active or repressive role of HP1 γ is determined and its nature and mechanisms. We hypothesised that the mode of HP1 γ regulation might be dependent on the ‘context’, that is which regulatory factors (including specific epigenetic modifications) it interacts with in different nuclear environments (e.g euchromatic or heterochromatic).

One obvious potential determinant of the function of HP1 γ is the histone mark H3K9me3 which has been shown to specifically interact with HP1 proteins and recruit them to the silent chromatin (Bannister, Zegerman et al. 2001, Lachner, O'Carroll et al. 2001, Jacobs and Khorasanizadeh 2002). Moreover, co-enrichment of HP1 γ and H3K9me3 has been observed in the coding regions of several active genes (Vakoc, Mandat et al. 2005) and implicated in the alternative splicing of a reporter gene (Saint-Andre, Batsche et al. 2011).

Another potential determinant of the function of HP1 γ is the histone variant H3.3. This idea originated from previous studies which showed specific interaction between H3.3 and HP1 γ but not HP1 α and β (Loyola, Bonaldi et al. 2006, Kim, Heo et al. 2011). Intriguingly, the binding of both H3.3 and HP1 γ to the promoter of the HSP70 genes was found to be interdependent, in which H3.3 binding required expression of HP1 γ and vice versa, and both were required for HSP70 gene expression (Kim, Heo et al. 2011). Moreover, differential enrichment of H3.3 on different chromosomal regions has been reported to be dependent on HP1 level in *Drosophila* (de Wit, Greil et al. 2007).

In this thesis, we set out to investigate whether co-enrichment of H3K9me3 or H3.3 with HP1 γ contributes to different gene expression outcomes *in vivo*. In order to reveal the genomic distribution pattern of these proteins and histone modification, chromatin immunoprecipitation followed by high throughput sequencing (ChIP-seq) were performed. By comparing the binding pattern between these factors and HP1 γ and correlating this with the RNA-seq data, genes or regions that are cooperatively, separately or independently regulated by these factors might be revealed. This study might also provide insight into the role of HP1 γ in gene regulation and identify genes that might be directly or indirectly regulated by HP1 γ .

3.3.1 H3.3 and H3K9me3 specifically associate with HP1 γ in MEFs

In order to confirm the association between HP1 γ with H3.3 or H3K9me3 in our cell model, co-immunoprecipitation experiments were performed in MEFs. In this experiment, lysed MEF nuclei were treated with micrococcal nuclease (MNase) followed by high salt (340mM NaCl) in order to enrich for histone proteins in the chromatin fraction as well as the closely-associated proteins (Figure S13 in appendix). Endogenous HP1 γ protein or H3.3B-EGFP fusion protein were pulled down using anti-HP1 γ antibody and anti-GFP antibody respectively. These ‘pulled-down’ fractions which contain HP1 γ or H3.3B-EGFP fusion protein and their associated protein partners were then tested by western blotting using specific antibodies. The efficiency of this experiment was firstly assessed by checking for the presence of HP1 γ protein in the pulled-down fraction generated using the anti-HP1 γ antibody by western blotting. Figure S14A in appendix showed strong enrichment of HP1 γ protein in the pull-down fraction (right lane). Also, HP1 γ protein was diminished in the unbound fraction (middle lane) indicating high efficiency of pull down (Figure S14A in appendix).

This pull-down fraction was then tested for the presence of histone H3, H3.3 and H3K9me3 with specific antibodies. Figure 3.22A shows that H3, H3.3 and H3K9me3 were all co-immunoprecipitated with HP1 γ (Figure 3.22A). However, interaction was not revealed with the IgG control. This result indicates the specific

interaction between HP1 γ and histone H3, H3.3 and H3K9me3 in our cell model.

I next ask whether H3.3 also interacts with other HP1 isoforms. Due to the lack of availability of efficient antibodies for pulling down HP1 α and HP1 β , GFP-antibody was employed to pull down H3.3B-EGFP fusion protein in the HP1 γ H3.3B-EGFP MEFs (see methods and material section 2.3.2.2 for details of this mouse model) and tested for the association with the different HP1 isoforms. Given that H3.3 proteins derived from the *H3f3a* (H3.3a) and *H3f3b* (H3.3b) genes have the same amino acid sequences and hence protein structure, this experiment should reflect the interaction between total H3.3 and HP1 isoforms.

As shown in the input lanes, weaker signal was revealed for both HP1 α and HP1 β relative to HP1 γ (Figure 3.22B). This difference can be explained by the difference in antibody efficiency. GFP-antibody pull-down fractions were tested by western blotting using anti-HP1 α , HP1 β or HP1 γ antibodies. Figure 3.22B shows that H3.3B-EGFP co-immunoprecipitated specifically with HP1 γ . A weak interaction was also revealed for HP1 α but not HP1 β (Figure 3.22B). Importantly, no signal was detected when performing this experiment in WT MEFs that do not express H3.3B-EGFP fusion protein, indicating that detected signal was specific (Figure S14B in appendix). Taken together, these results indicate the specific association between HP1 γ with histone H3, H3.3 as well as the histone H3K9me3 in MEFs.

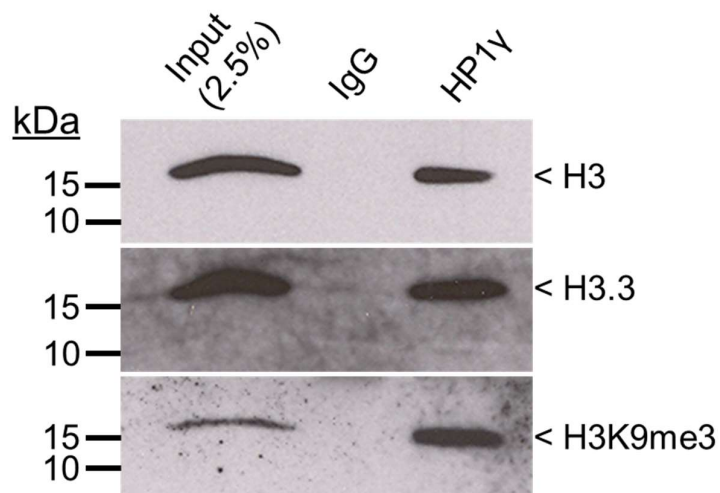
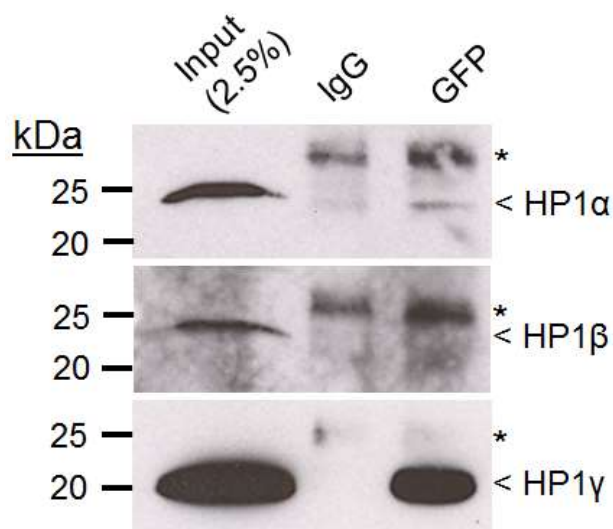
A**B**

Figure 3.22 Co-immunoprecipitation analysis of HP1γ with histone H3, H3K9me3 and H3.3. (A) Histone H3, H3.3 and H3K9me3 were all co-immunoprecipitated with endogenous HP1γ. No interaction was revealed with IgG control. (B) H3.3B-EGFP co-immunoprecipitated specifically with endogenous HP1γ. Weak interaction was also observed between H3.3B-EGFP and HP1α. Asterisks indicate IgG bands.

3.3.2 H3.3 and H3K9me3 co-localise with HP1 γ in MEFs

Immunofluorescence microscopy was employed to visualise the sites of interaction between HP1 γ , H3.3 or H3K9me3 in WT MEFs. Specific antibody targeting endogenous HP1 γ and H3K9me3 were used whereas anti-GFP antibody was used for visualising the H3.3B-EGFP fusion proteins. For a more accurate analysis of the subnuclear co-localisation of these proteins, confocal microscopy which provides images of higher optical resolution and contrast was employed.

Consistent with previous presented data in section 3.2.2, two different staining patterns of HP1 γ were revealed in WT MEFs nuclei. HP1 γ was found enriched in DAPI-dense regions characteristics of heterochromatin clusters in a subset of cells (WT(1)) while the others showed diffuse staining of HP1 γ (WT(2)) (Figure 3.23 and Figure 3.24, left two column). Staining of H3K9me3 in WT MEFs showed clear and distinct co-localisation with DAPI-dense regions (Figure 3.23) as previously described (Figure 3.17 in section 3.2.2). H3K9me3 and HP1 γ were found co-localised predominately in the DAPI-dense regions of WT MEFs nuclei. As presented in section 3.2.2 (Figure 3.17), no obvious change in H3K9me3 was observed in response to knockout of HP1 γ (Figure 3.23, right two column).

In WT MEFs, H3.3B-EGFP was found predominately in euchromatin regions and excluded from DAPI-dense regions in most cases (Figure 3.24). Similar pattern has also been observed for endogenous H3.3B-EGFP without anti-GFP antibody staining (Figure S15 in appendix). Distribution of H3.3 and HP1 γ are not excluded from each other and sites with high levels of both H3.3 and HP1 γ co-localisation were observed (Figure 3.24). These co-localisation sites were found predominately in euchromatic regions but not in DAPI-dense regions (Figure 3.24, indicated by red arrows in enlarged images). In response to HP1 γ deletion, no significant changes in H3.3B-EGFP distribution were observed (Figure 3.24).

Taken together, this experiment suggests that interaction between HP1 γ and H3K9me3 or H3.3 take place in specific subnuclear compartments. Removal of HP1 γ does not lead to significant changes of H3K9me3 and H3.3 localisation at least at the resolution of microscopic analysis. However, further experiments are

required to get a better insight into whether HP1 γ affects the distribution of H3K9me3 and H3.3 at the genomic level.

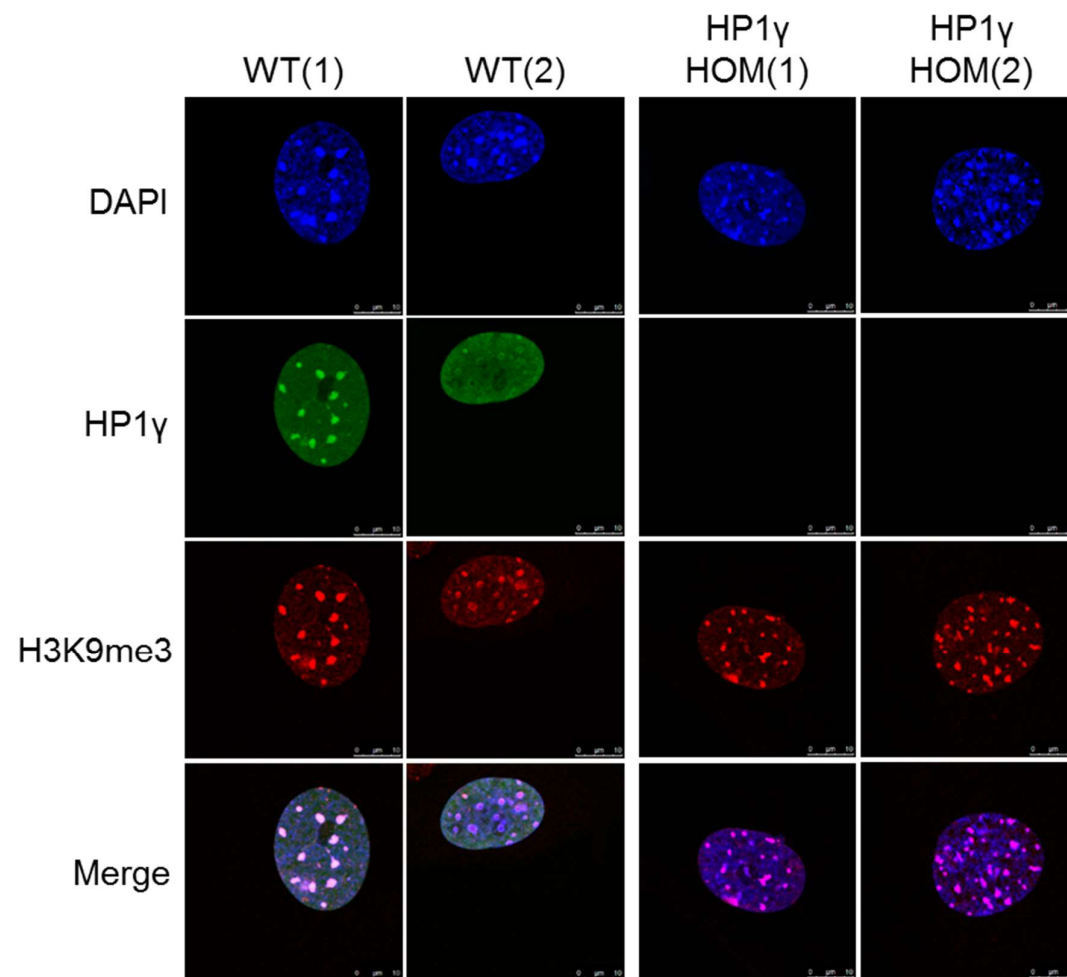


Figure 3.23 Immunofluorescence analysis of HP1 γ and H3K9me3 co-localisation in HP1 γ WT and HOM MEFs. Two different staining patterns of HP1 γ were observed. Enrichment of HP1 γ in DAPI-dense regions as was shown in a subset of nuclei (WT(1), first left column) while other nuclei showed diffuse staining of HP1 γ (WT(2), second left column). H3K9me3 showed clear and distinct co-localisation with DAPI-dense regions in WT MEFs. HP1 γ and H3K9me3 co-localise predominately at DAPI-dense regions in WT MEFs. Upon knockout of HP1 γ , no significant change of H3K9me3 was observed. Scale bar, 10 μ m. These images were generated by Leica confocal microscope and analysed with Leica LAS AF Lite Software.

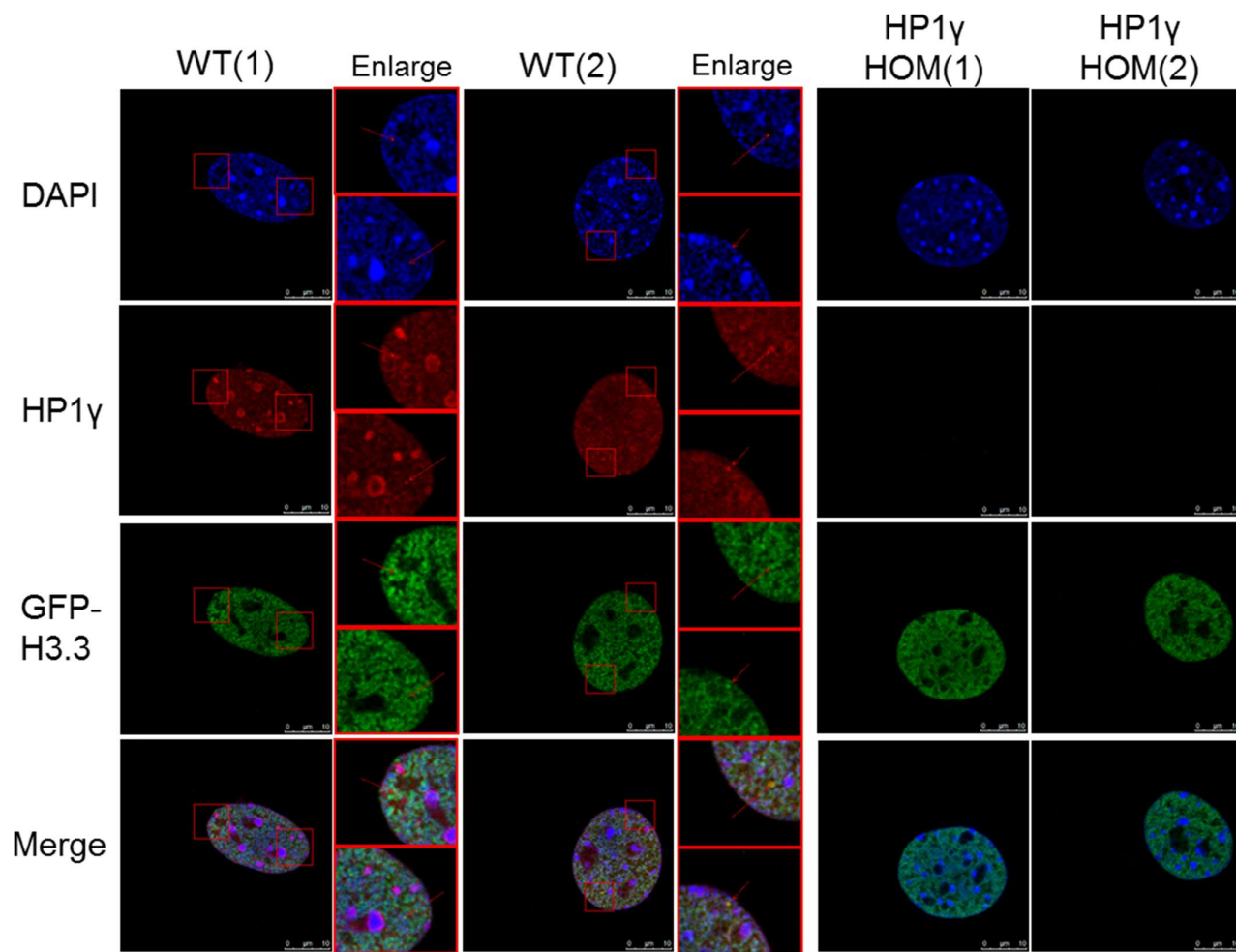


Figure 3.24 Immunofluorescence analysis of HP1 γ and H3.3B-EGFP co-localisation in WT and HOM HP1 γ H3.3B-EGFP MEFs. Two different staining patterns of HP1 γ were observed. Enrichment of HP1 γ in pericentric (DAPI-dense) regions were shown in a subset of nuclei (WT(1), first left column) while other nuclei showed diffuse staining of HP1 γ (WT(2), second left column). H3.3B-EGFP was found predominately in euchromatin regions. Co-localisation of HP1 γ and H3.3 were observed predominately in euchromatic regions but not in DAPI-dense regions. Upon knockout of HP1 γ no significant change of H3K9me3 was observed. Enlarged parts of the nuclei (indicated by the red boxes) were shown on the right of left two rows for WT(1) and WT(2). Regions of strong co-localisation of HP1 γ and H3.3 were indicated by the red arrows. Scale bar, 10 μ m. These images were generated by Leica confocal microscope and analysed with Leica LAS AF Lite Software.

3.3.3 Chromatin immunoprecipitation (ChIP) for localising HP1 γ , H3K9me3 and H3.3B-EGFP in the MEF's genome

In order to gain a better insight into the distribution of HP1 γ , H3K9me3 and H3.3 in the genome, chromatin immunoprecipitation (ChIP) was performed. Each ChIP experiment using specific targeting antibody was validated by examining target protein enrichment on specific genomic regions by qRT-PCR.

Anti-HP1 γ and anti-H3K9me3 antibodies were used to target endogenous HP1 γ and H3K9me3 in MEFs. As presented in section 3.2.2, enrichment of HP1 γ was observed on major satellite (Mj-sat) repeat elements which agrees with the immunofluorescence experiment in which localisation of HP1 γ was detected in both heterochromatic and euchromatic regions (Figure 3.16 in section 3.2.2, Figure 3.23 and 3.24 in section 3.3.2). Enrichment of HP1 γ has also been confirmed on the region 4kb downstream of the transcription start site of the *Gapdh* gene (Gapdh+4K) as reported by Vakoc *et al* previously (Vakoc, Mandat et al. 2005) (Figure 3.25). Knockout of HP1 γ caused significant reduction of HP1 γ enrichment on these regions indicating the specificity of the anti-HP1 γ antibody used. Level of HP1 γ revealed in HP1 γ HOM MEFs would therefore provide a measure of background.

Similar results were obtained in the H3K9me3 ChIP experiment where it was found to be enriched on Mj-sat and the Gapdh+4K region (Figure 3.26) This observation is again consistent with the previously presented immunofluorescence data showing co-localisation of H3K9me3 with DAPI-dense regions (Figure 3.17 in section 3.2.2, Figure 3.23 in section 3.3.2) as well as a previous published study (Vakoc, Mandat et al. 2005). Observed enrichment of both H3K9me3 and HP1 γ indicates that H3K9me3 provides potential binding sites for HP1 γ in these regions. Enrichment of H3K9me3 has also been examined on the promoters of silent (*Neurod6* and *Myl3*) and active (EF1 α and *Gapdh*) genes. As one might expect, a relatively high enrichment of the silencing mark H3K9me3 was revealed on the *Neurod6* and *Myl3* genes which are silent in fibroblasts while a relatively low enrichment of H3K9me3 was found on the housekeeping genes EF1 α and *Gapdh* (Figure 3.26).

As a specific antibody targeting endogenous H3.3 for ChIP became available only

very recently and has a low efficiency (Figure S16 in appendix), therefore an anti-GFP antibody was employed to reveal the distribution pattern of the H3.3B-EGFP fusion protein in the WT HP1 γ H3.3B-EGFP MEFs (see methods and material section 2.3.2.3 for details of the mouse model). The specificity of this antibody was first tested by comparing results obtained from WT CBA or HP1 γ H3.3B-EGFP MEFs which express the H3.3B-EGFP fusion protein. As shown in Figure 3.27A, H3.3B-EGFP was enriched on the promoter of the expressed housekeeping genes EF1 α and *Gapdh* (Figure 3.27A), consistent with a previously published study (Harada, Okada et al. 2012). In contrast, the signal for H3.3B-EGFP was greatly reduced in WT CBA MEFs on this region indicating the specificity of this antibody for the ChIP experiment (Figure 3.27A).

Binding of H3.3B-EGFP was then further analysed on different genomic regions including the promoter of the silent genes *Neurod6* and *Myl3* as well as the Mj-sat repeat element. As expected, a low level of H3.3B-EGFP was revealed on Mj-sat (Figure 3.27C) which is in line with the previously presented immunofluorescence data showing that H3.3B-EGFP is largely excluded from DAPI-dense regions (Figure 3.24 section 3.3.2). Consistent with this was the recent finding of low levels of H3.3 on Mj-sat in mESC and *neural progenitor cells* (NPC) (Elsasser, Noh et al. 2015). Unexpectedly, the experiment shown here showed a high level of H3.3B-EGFP on the promoter of the silent genes *Neurod6* and *Myl3* compared to the transcriptionally active EF1 α and *Gapdh* genes (Figure 3.27C) relative to total input. This result may be explained by the fact that the promoter regions of the actively transcribed genes are more susceptible to the shearing energy during the sonication step of the ChIP experiment due to their open structure and low nucleosome occupancy. As a consequence, chromatin fragments derived from these regions were enriched in the input material. Importantly, as histone H3.3 is a component of the nucleosome, normalising H3.3B-EGFP signal to total input may not reflect the proportion of nucleosomes containing H3.3B-EGFP in these regions. In order to estimate the proportion of H3.3B-EGFP containing nucleosomes on these regions, ChIP using anti-H3 antibody on the same chromatin samples was performed and used as a normalisation control.

As expected, the level of histone H3 which represents the nucleosome occupancy was found to be diminished at the active genes *EF1 α* and *Gapdh* whereas a high level of H3 was revealed on the silent genes *Neurod6* and *Myl3* as well as at the pericentric Mj-sat repeats (Figure 3.27B). High levels of H3.3B-EGFP were revealed on the promoters of the active genes *EF1 α* and *Gapdh* relative to H3 (Figure 3.27D). In contrast, silent genes *Neurod6* and *Myl3* as well as the pericentric Mj-sat repeats showed low levels of H3.3B-EGFP relative to H3 (Figure 3.27D). This result indicates that there is a high proportion of H3.3 on the promoter of the active genes tested while that of the silent genes and Mj-sat elements was relatively low.

Taken together, results obtained from the ChIP experiments using different antibodies agree with previous published studies (Vakoc, Mandat et al. 2005, Harada, Okada et al. 2012) as well as the data obtained from immunofluorescence microscopy. The ChIP material generated was therefore suitable for interrogation by high-throughput sequencing to reveal the genome-wide distribution pattern of these proteins.

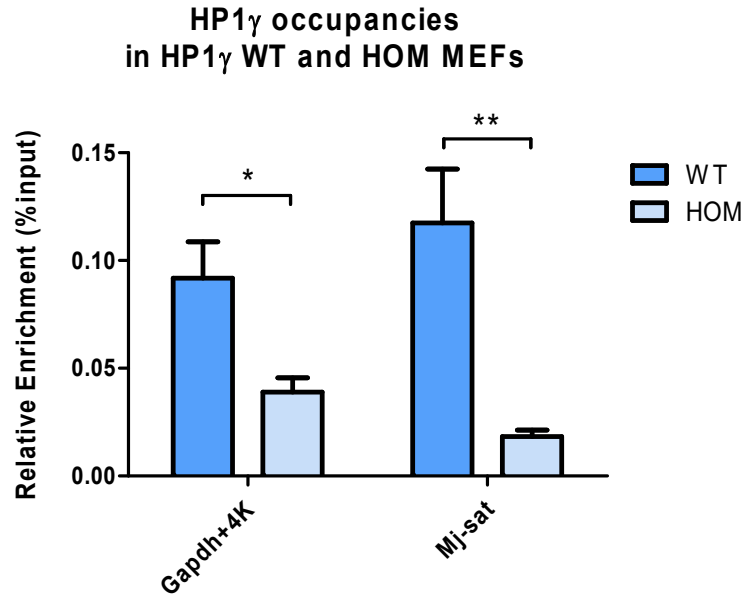


Figure 3.25 Relative enrichment of HP1 γ on various genomic regions in HP1 γ WT and HOM MEFs as revealed by ChIP. Enrichment of HP1 γ was observed on major satellite (Mj-sat) repeat elements and on the region 4kb downstream of the transcription start site of the *Gapdh* gene (Gapdh+4K). Deletion of HP1 γ caused significant reduction of HP1 γ enrichment on these regions indicating the specificity of the anti-HP1 γ antibody used. Level of HP1 γ revealed in HP1 γ HOM MEFs represents the background noise of the experiment. Student's t-test: *P<0.05, **P<0.01 (compared to WT after being normalised to input signal). Error bars: SEM. n $\geq$ 10.

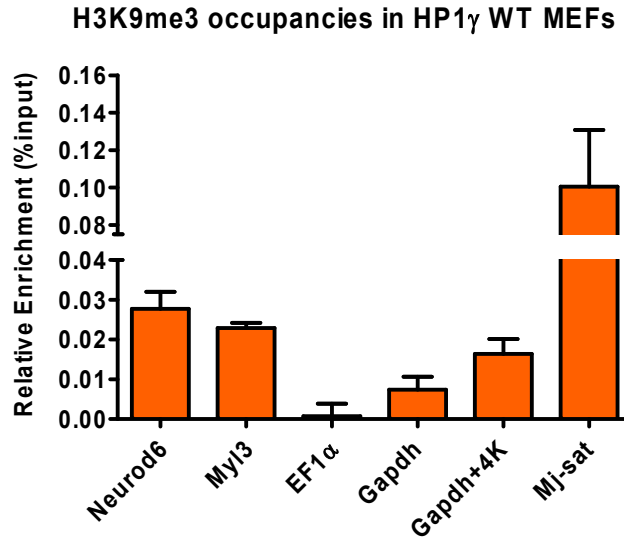


Figure 3.26 Relative enrichment of H3K9me3 on various genomic regions in HP1 γ WT MEFs as revealed by ChIP. Enrichment of H3K9me3 was observed on silent genes *Neurod6* and *Myl3* and the Mj-sat repeat element. Low level of H3K9me3 was revealed on EF1 α and *Gapdh* promoter which are actively expressed housekeeping genes. Moreover, enrichment of H3K9me3 has also been observed on the region 4kb downstream of the *Gapdh* gene (Gapdh+4K) which is consistent with a previously published study (Vakoc, Mandat et al. 2005). Error bars: SEM. n=6.

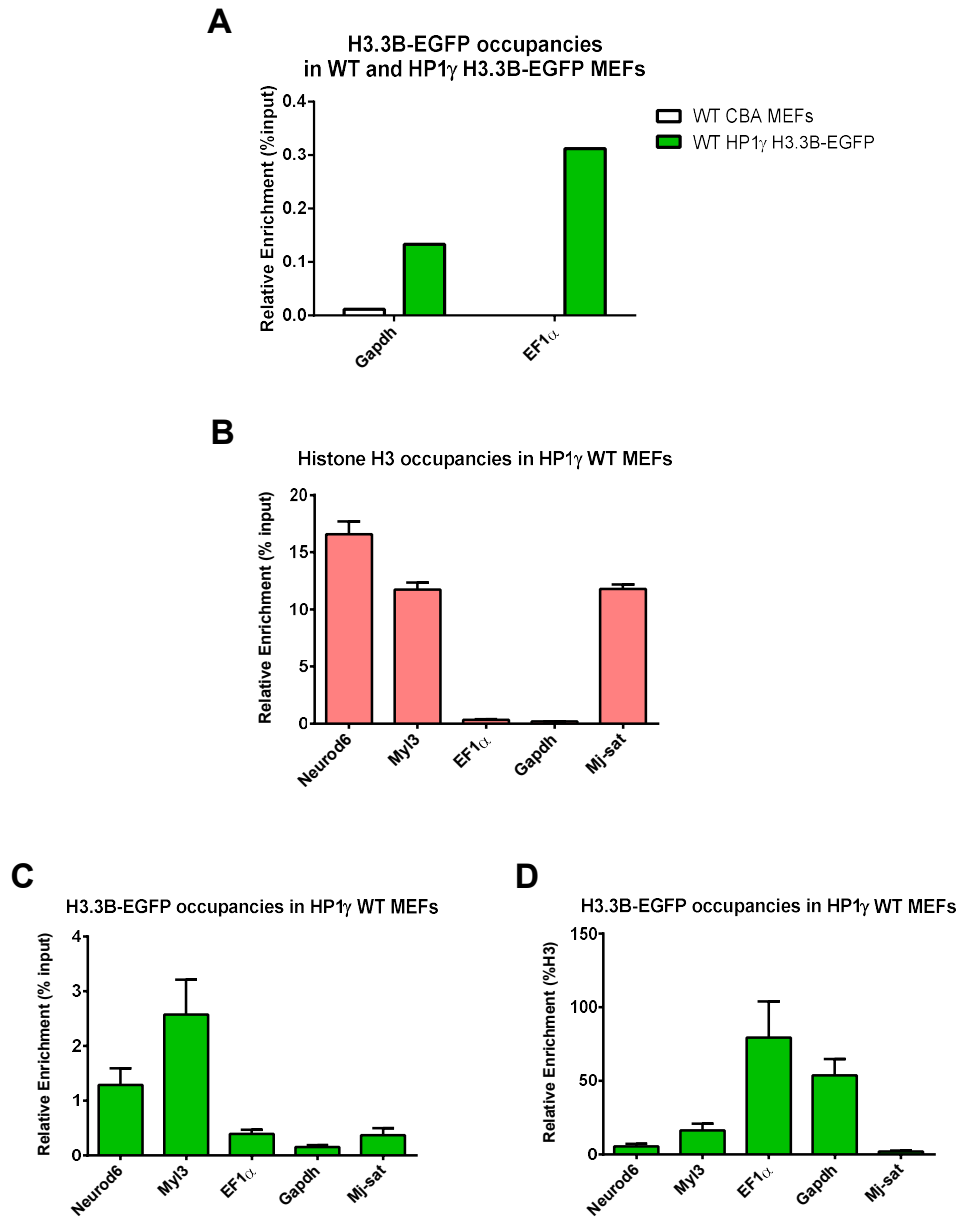


Figure 3.27 Relative enrichment of H3.3B-EGFP on various genomic regions in WT HP1 γ H3.3B-EGFP MEFs as revealed by ChIP. (A) H3.3B-EGFP signal was obtained from the transcriptionally active housekeeping genes *EF1 α* and *Gapdh* in HP1 γ H3.3B-EGFP MEFs which express the H3.3B-EGFP fusion protein. Low signal on these regions were obtained in the WT CBA MEFs indicating the specificity of the anti-GFP antibody used in this ChIP experiment. $n=2$ (qRT-PCR technical replicates). (B) Level of histone H3 which represents the nucleosome occupancy was diminished at the promoter of active genes *EF1 α* and *Gapdh* and high in silent genes *Neurod6* and *Myl3* as well as the *Mj-sat*. Error bars: SEM. $n=4$. (C) H3.3B-EGFP was enriched on the promoters of silent genes *Neurod6* and *Myl3* and diminished on the active genes *EF1 α* and *Gapdh* as well as *Mj-sat* relative to total input. Error bars: SEM. $n=10$. (D) High levels of H3.3B-EGFP were revealed

on the promoters of the active genes *EF1 α* and *Gapdh* relative to H3 indicating the high proportion of H3.3B-EGFP containing nucleosomes in these regions. Error bars: SEM. n=3.

3.3.4 Removal of HP1 γ did not significantly change the level of H3K9me3 and H3.3B-EGFP on selected gene regions

Having shown the reliability of the ChIP experiment in the previous section, I then asked whether H3K9me3 or H3.3B-EGFP occupancies on the regions tested were changed upon HP1 γ knockout. As shown in Figure 3.28A and B, deletion of HP1 γ caused an apparent reduction of H3K9me3 in all regions tested except the *EF1 α* promoter. However, as the signal of H3K9me3 on *EF1 α* was very near to background and thus subject to increased error which likely resulted in an erroneous fold change as reflected by the lack of statistical significance. The changes in H3K9me3 enrichment of other regions tested were also not individually statistically significant except at the inactive gene *Myl3* (Figure 3.28A and B).

HP1 γ deficiency also resulted in a reduction of H3.3B-EGFP at all regions examined. The relative fold change of H3.3B-EGFP enrichment was minimal at *Mj-sat* and small in the two inactive genes *Neurod6* and *Myl3* while bigger effects were observed in *Gapdh* and *EF1 α* which are transcriptionally active. However, none of these detected changes were statistically significant (Figure 3.28C and D).

Taken together, these results showed that removal of HP1 γ has apparently only minor effects on distribution of H3K9me3 and H3.3B-EGFP protein on the regions tested. In order to have a better idea of whether this will be affected in other regions of the genome, ChIP samples generated here were analysed by high throughput sequencing (ChIP-seq) (see sections 3.3.5).

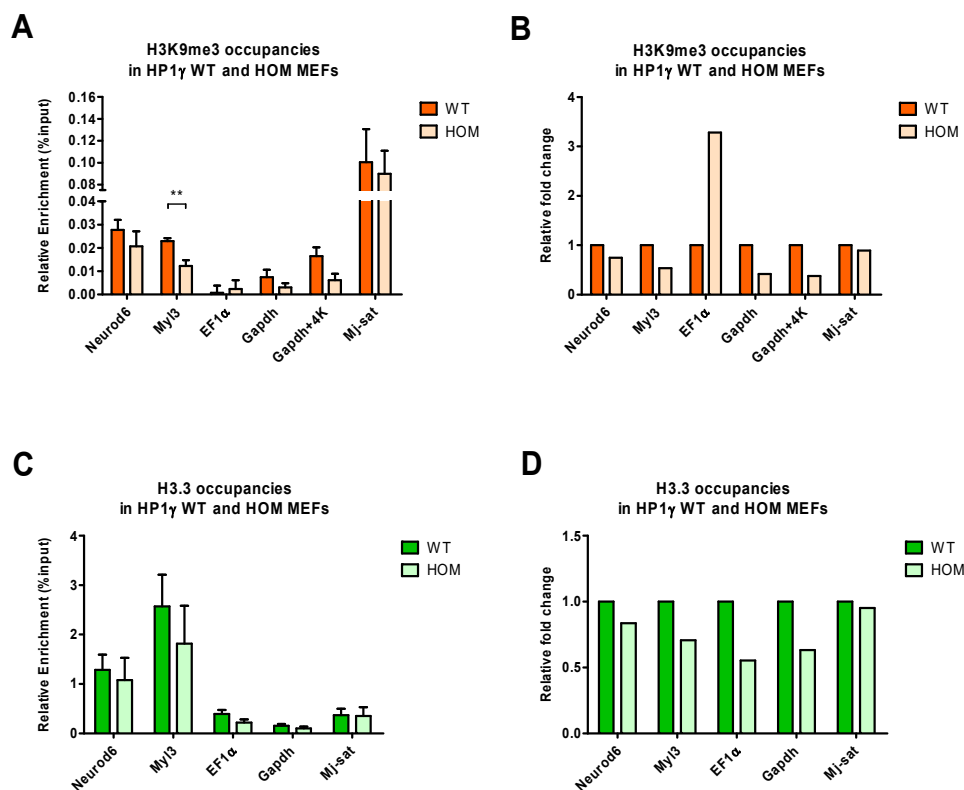


Figure 3.28 Relative enrichment of H3K9me3 and H3.3B-EGFP on various genomic regions in HP1γ WT and HOM MEFs. (A) Except EF1α, all of the regions tested showed reduction of H3K9me3 in response to HP1γ knockout. However, only *Myl3* showed a statistically significant change in level of H3K9me3 relative to WT. Student's t-test: **P<0.01 (compared to WT after being normalised to input signal). Error bars: SEM. n≥5. (B) Relative fold change of H3K9me3 signal detected on each region in response to HP1γ knockout relative to WT. (C) All regions examined showed reduction of level of H3.3B-EGFP upon HP1γ knockout. However, none of these changes were statistically significant. Error bars: SEM. n≥8. (D) Relative fold change of H3.3B-EGFP signal detected on each region in response to HP1γ knockout relative to WT.

3.3.5 Investigation into the effect of HP1 γ deletion in H3.3B-EGFP distribution genome wide

As mentioned earlier, histone variant H3.3 is a potential determinant of the function of HP1 γ suggested by their specific interaction (Figure 3.22) as well as their observed cooperation in allowing expression of the HSP70 genes as discussed above (Loyola, Bonaldi et al. 2006, Kim, Heo et al. 2011). In order to get a better insight into the effect of HP1 γ in H3.3 distribution, ChIP-seq using anti-GFP antibody which targets the H3.3B-EGFP was performed in both HP1 γ WT and HOM MEFs.

The general distribution patterns of H3.3B-EGFP are very similar in HP1 γ WT and HOM MEFs. As shown in Figure 3.29, pronounced peak of enrichment around the TSS as well as the transcription end site (TES) were observed (Figure 3.29). Interestingly, in both WT and HOM MEFs H3.3B-EGFP showed a dip at the TSS. These observation is consistent with a previously published study in both mESCs and NPCs (Goldberg, Banaszynski et al. 2010).

In order to determine whether H3.3B-EGFP enrichment correlates with gene activity, the genes in MEFs were grouped in four tiers by expression level and tested for their level of H3.3B-EGFP by ChIP-seq. High level of binding of H3.3B-EGFP was observed in both HP1 γ WT and HOM MEFs on highly expressed genes (Figure 3.30, Fourth Quartile) while the lowest expressed genes have a relatively low level of H3.3B-EGFP. In WT MEFs, enrichment of H3.3B-EGFP was observed at the TES as well as the regions around TSS at a relatively lower level on the top quartile of highly expressed genes (Figure 3.30, Fourth Quartile). There is a dip at the TSS as shown previously (Figure 3.29) and is consistent with a study in murine ESCs (Goldberg, Banaszynski et al. 2010). Interestingly, both the peak with this central dip at the TSS was preserved on the highest expressed genes (Fourth Quartile) but lost on the lowest expressed genes (Figure 3.30, First Quartile). Surprisingly, in response to HP1 γ knockout, enrichment of H3.3B-EGFP at the region around the TSS was increased on the top quartile of highly expressed genes (Figure 3.30, Fourth Quartile). A summary of statistical analysis on the TES to TSS binding ratio of H3.3B-EGFP is shown in table 3.4. Thus, particularly in highly expressed genes

it seems that HP1 γ rather than recruiting H3.3 appears to inhibit its incorporation. Given that surprising nature of this observation, and the fact that the active promoters looked at by qRT-PCR showed a reduction in H3.3 following HP1 γ knockout it would be important to confirm the result with an independent biological replicate.

In order to investigate whether HP1 γ sensitive genes have differential H3.3B-EGFP binding, distribution of H3.3B-EGFP were examined separately for genes that were upregulated, downregulated or not significantly changed upon HP1 γ knockout. In HP1 γ WT MEFs, the overall distribution patterns of H3.3B-EGFP were very similar among the three groups of genes in which pronounced enrichment of H3.3B-EGFP was observed in both TSS and transcriptional end site (TES) (Figure 3.31). Genes that were upregulated upon HP1 γ knockout showed a higher level of enrichment of H3.3B-EGFP in general (Figure 3.31). Genes whose expression level was not changed upon HP1 γ knockout showed enrichment of H3.3B-EGFP with a very similar level to the upregulated genes (Figure 3.31). In contrast, the genes that were downregulated by HP1 γ knockout showed a similar pattern of H3.3 but at a lower level than the upregulated genes.

In HP1 γ HOM MEFs, again we observed similar overall distribution patterns of H3.3B-EGFP among the three groups of genes with pronounced enrichment of H3.3B-EGFP at both TSS and TES (Figure 3.31). Also, higher enrichment of H3.3B-EGFP was observed on both the unchanged genes and the upregulated genes compared to downregulated genes (Figure 3.31). Interestingly, in response to HP1 γ knockout, a more pronounced enrichment of H3.3B-EGFP was observed at the regions around TSS. This indicates that HP1 γ seems to prevent H3.3 loading at the TSS in all three subsets of these genes. A representative genome browser track for gene *Gpr165* is also shown (Figure 3.32). However, it is possible that the increased H3.3 at the TSS in the upregulated genes might contribute to the upregulation by influencing transcriptional initiation. As this enrichment for H3.3 at the TSS occurs on all the subsets it is unlikely to be simply a consequence of altered transcription.

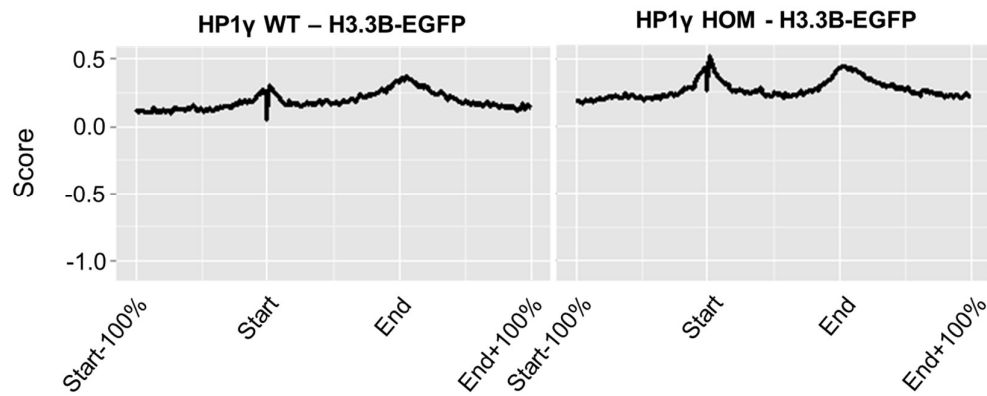


Figure 3.29 Average profile plot for H3.3B-EGFP enrichment in HP1 γ WT and HOM MEFs. X-axis shows the normalised length where ‘Start’ indicates that transcription start site (TSS) and ‘End’ indicates the transcription end site (TES). Y-axis shows the coverage in windows. Data is shown as a proportion of input. HP1 γ WT (left panel) and HOM (right panel) MEFs showed very similar distribution pattern of H3.3B-EGFP in which pronounced peaks of enrichment were observed around TSS as well as TES. A central dip at the TSS was shown in both HP1 γ WT and HOM MEFs. n=1.

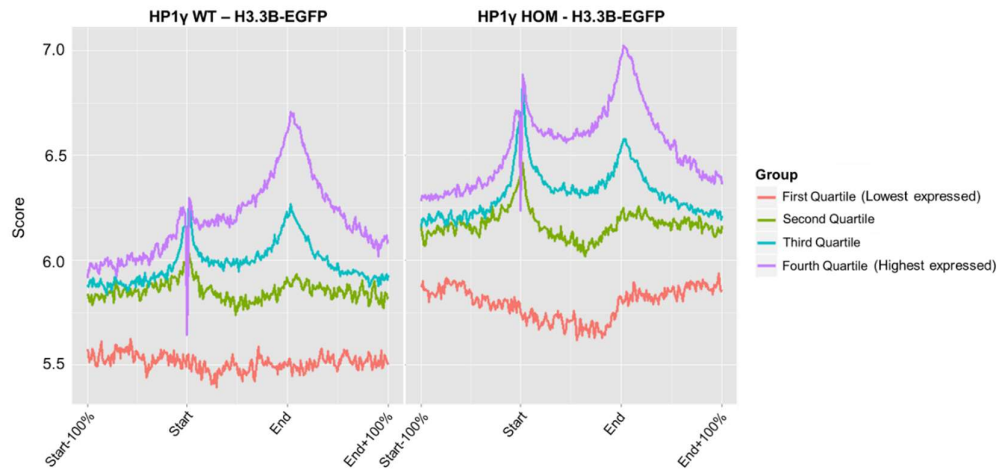


Figure 3.30 Average profile plot for H3.3B-EGFP of genes grouped into four tiers by expression level in HP1 γ WT and HOM MEFs. X-axis shows the normalised length where ‘Start’ indicates that transcription start site (TSS) and ‘End’ indicates the transcription end site (TES). Y-axis shows the coverage in windows. Distribution pattern of HP1 γ and H3K9me3 to genes with different expression level were revealed. ‘First Quartile’ represents genes with lowest expression level while “Fourth Quartile” represents genes with highest expression level. Data is shown as a proportion of input. In both HP1 γ WT and HOM MEFs, high level of H3.3B-EGFP enrichment was observed on highly expressed genes. High level of binding of H3.3B-EGFP was observed in both HP1 γ WT and HOM MEFs on highly expressed genes (Fourth Quartile) while lowest expressed genes have relatively low level of H3.3B-EGFP. The central dip at TSS was preserved on the highest expressed genes (Fourth Quartile) but lost on the lowest expressed genes (First Quartile). In HP1 γ HOM MEFs, enrichment of H3.3B-EGFP at the region around the TSS was increased on the top quartile of highly expressed genes. n=1.

Group	TES to TSS ratio	P-value
WT Fourth Quartile	0.155	1.33×10^{-6}
HOM Fourth Quartile	0.409	4.03×10^{-6}

Table 3.4 A summary of statistical analysis on the TES to TSS binding ratio of H3.3B-EGFP. Wilcox test.

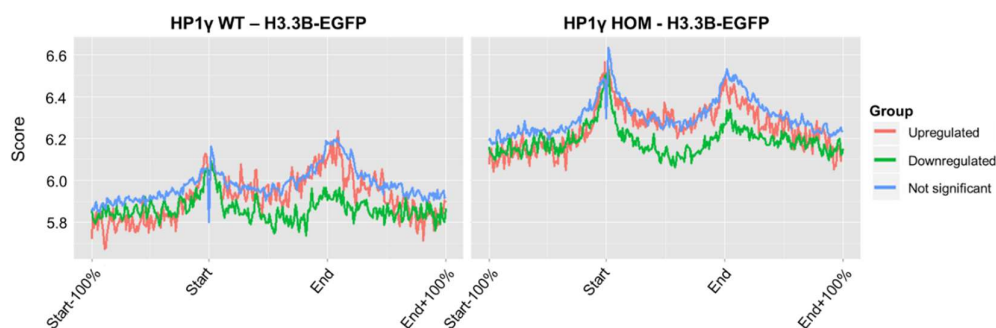


Figure 3.31 Average profile plot for H3.3B-EGFP of genes differentially expressed in HP1 γ knockout in HP1 γ WT and HOM MEFs. X-axis shows the normalised length where ‘Start’ indicates that transcription start site (TSS) and ‘End’ indicates the transcription end site (TES). Y-axis shows the coverage in windows. Data is shown as a proportion of input. The overall distribution patterns of H3.3B-EGFP were very similar among the three groups of genes in both HP1 γ WT (left panel) and HOM (right panel) MEFs in which pronounced enrichment of H3.3B-EGFP was observed in both TSS and TES. In response to HP1 γ knockout (right panel), a more pronounced enrichment of H3.3B-EGFP was observed at the regions around TSS. n=1.

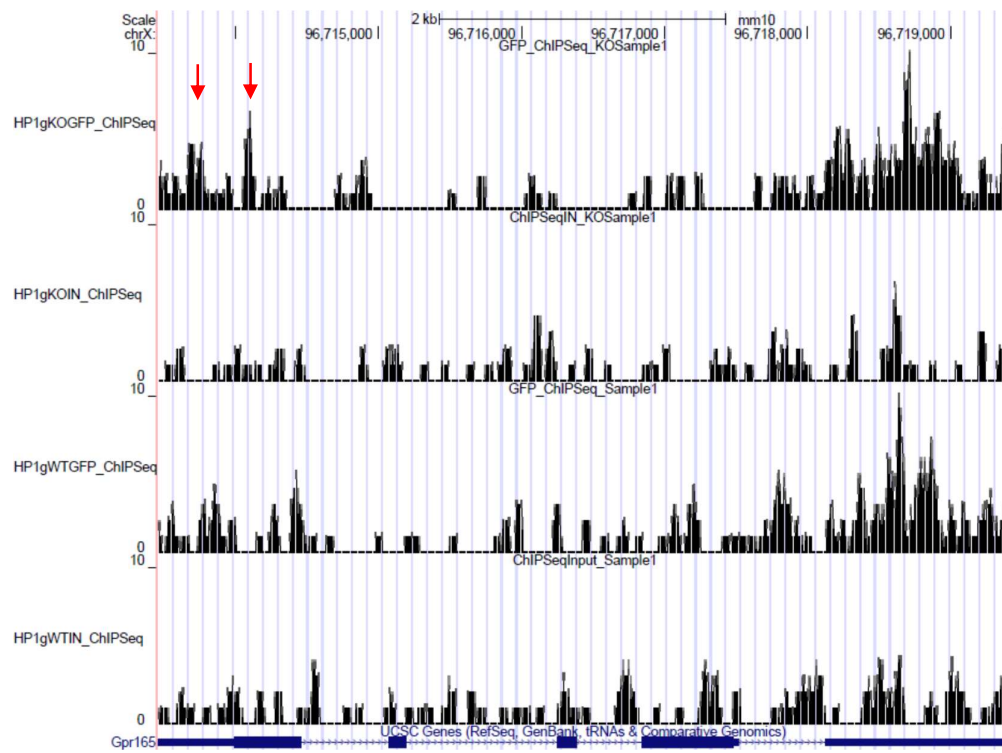


Figure 3.32 Genome browser view of representative gene *Gpr165*. *Gpr165* showed increased H3.3B-EGFP binding upon HP1 γ knockout (as indicated by red arrows).

3.3.6 Summary of result section 3.3

This section focused on investigation in the potential ‘context’ (H3K9me3, H3.3) which may determine the mode of function of HP1 γ . The main findings were as follows:

1. HP1 γ is specifically associated with histone H3, H3.3 as well as H3K9me3 in MEFs as shown by co-immunoprecipitation.
2. Removal of HP1 γ does not lead to significant changes of H3K9me3 and H3.3 localisation at the resolution of microscopic analysis.
3. Preliminary data reveal that deficiency of HP1 γ caused an increase in H3.3B-EGFP enrichment at the TSS.

3.4 Investigation into *FXN* silencing in Friedreich's Ataxia

In FRDA, patients carry GAA-TCC DNA triplet repeats with sizes ranging from 90 to 1000 repeats in intron 1 of the *FXN* gene which leads to *FXN* silencing. Data implicating epigenetic mechanisms in silencing of the *FXN* gene in FRDA have been provided by a number of studies (for details see introduction section 1.8) including three from our group (Saveliev, Everett et al. 2003, Chan, Torres et al. 2013, Libri, Yandim et al. 2014). In these studies, I showed that the silencing histone marks H3K9me3 and H3K27me3 were enriched on the *FXN* gene with pathological GAA triplet-repeat expansions in FRDA primary lymphocytes relative to healthy control (Figure 3.33B). Interestingly, treatment of these cells with the class III HDAC inhibitor nicotinamide was able to reduce these silencing histone marks at the *FXN* gene locus in FRDA (Figure 3.33B). A similar result was also obtained from the cerebellum of a mouse model which carries the pathological human frataxin (*hFXN*) transgene (Figure 3.33C). Moreover, histone H3 and H4 acetylation at the *FXN* gene locus was increased upon nicotinamide treatment in FRDA primary lymphocytes (Figure 3.33D) (Chan, Torres et al. 2013). Intriguingly, oral intake of nicotinamide was able to increase both *FXN* mRNA and protein level towards asymptomatic carriers' level and significantly reduce the level of H3K9me3 on the *FXN* gene in primary lymphocytes derived from FRDA patients (Figure 3.34) (Libri, Yandim et al. 2014). Taken together, these findings suggested that silencing of the *FXN* gene in FRDA is at least in part regulated by epigenetic mechanisms. Importantly, these data suggested that the silencing of *FXN* gene can be reversed by modulating the protein factors that play a role in epigenetic mechanisms.

As introduced in section 1.6, position effect variegation (PEV) is a biological phenomenon that describes the stochastic silencing of a gene located abnormally close to heterochromatin in a proportion of cells. This process is modulated by PEV genetic modifiers which are suppressors in a dosage dependent manner whereby their overexpression enhances gene silencing and downregulation causes reduced silencing (Festenstein, Sharghi-Namini et al. 1999, Festenstein and Kioussis 2000). It has been shown in previous studies of our group that PEV occurs when the affected gene is in close proximity to classical constitutive heterochromatin which

is composed of repetitive DNA elements (Reviewed in - Dillon and Festenstein 2002), as well as CTG and GAA pathological triplet-repeat expansions which occur in the human diseases, Myotonic Dystrophy and Friedreich's Ataxia (FRDA) respectively (Saveliev, Everett et al. 2003).

Furthermore, the extent of GAA-repeat induced silencing in mammals can be modulated *in vivo* by changing the dose of HP1 β , a reader of H3K9me3. This result implicated HP1 β in pathological silencing of the *FXN* gene and therefore as a potential disease modifier (Saveliev, Everett et al. 2003).

In line with this finding, a previously published study showed in cell culture where HP1 γ was significantly enriched on the region between the transcription start site and exon 1 of the *FXN* gene in an FRDA fibroblast cell line compared to healthy control (De Biase, Chutake et al. 2009) These results prompted us to investigate whether the aberrantly silenced *FXN* gene in FRDA can be reversed by modulating the reader of H3K9me3, HP1 γ . This project would potentially enhance our understanding of the pathophysiological mechanisms of the expanded DNA triplet repeats in mediating aberrant silencing of the *FXN* gene in FRDA.

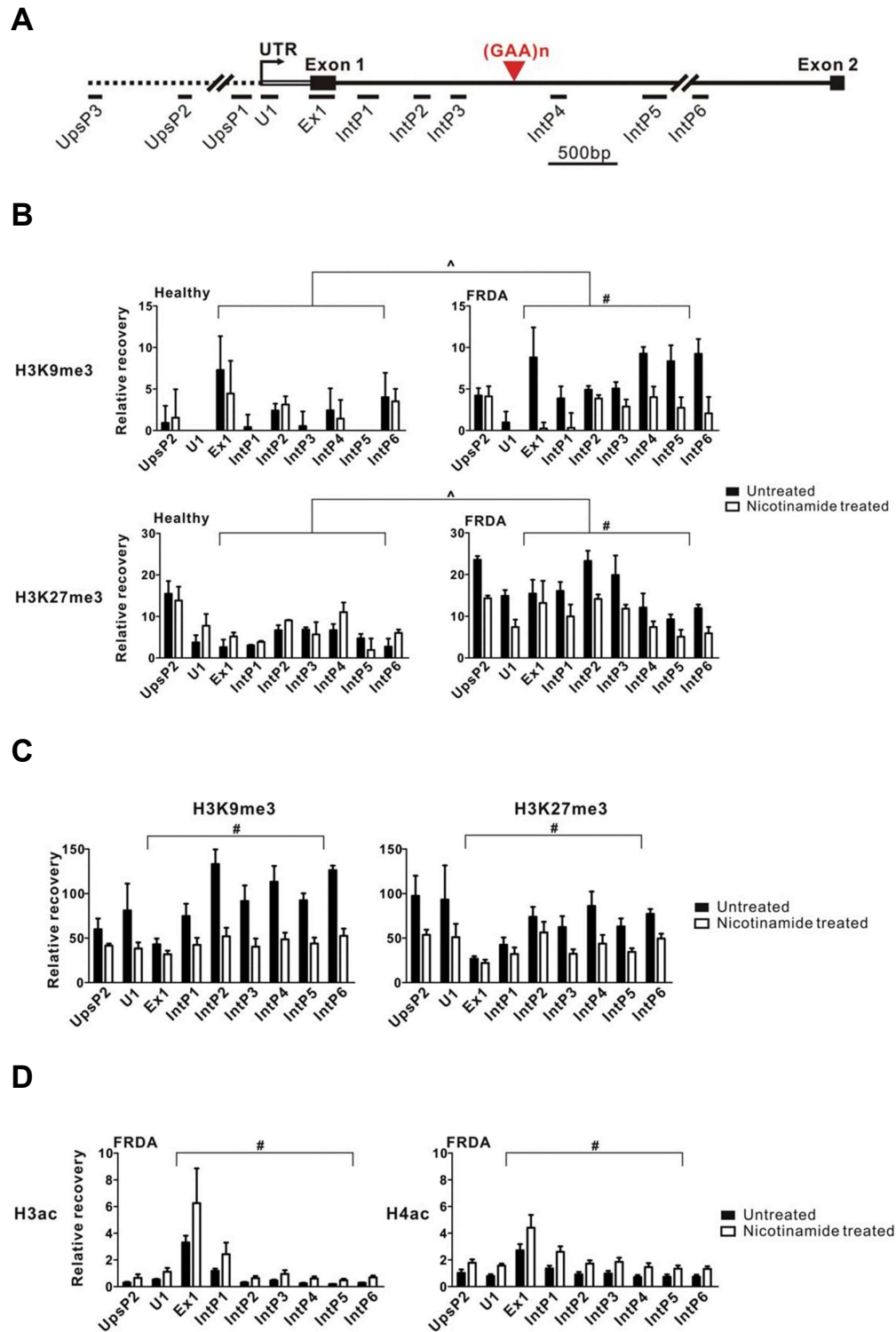


Figure 3.33 Silencing histone marks are enriched on the *FXN* gene in FRDA and can be reduced by treatment of nicotinamide. (A) Schematic representation of the *FXN* gene locus. The position and name of the primer sets used are indicated. (B) Silencing histone marks H3K9me3 and H3K27me3 were enriched on the *FXN* gene in FRDA primary lymphocytes relative to healthy control. Treatment of these cells

with nicotinamide reduced these silencing histone marks on the *FXN* gene relative to untreated control. (C) Six days treatment of nicotinamide was able to reduce silencing histone marks on the pathological h*FXN* transgene in the cerebella of the transgenic mouse. (D) Treatment of nicotinamide increased histone H3 and H4 acetylation level on *FXN* gene in FRDA primary lymphocytes relative to untreated control. There was a collaborative study in the lab from design and optimisation of ChIP to performing the definitive experiments. Panel D was entirely my experiment whereas I contributed to the other panels by preparing chromatin samples. This figure is adapted by permission from Oxford University Press: Human Molecular Genetics “Chan, P. K., R. Torres, C. Yandim, **P. P. Law**, S. Khadayate, M. Mauri, C. Grosan, N. Chapman-Rothe, P. Giunti, M. Pook and R. Festenstein (2013). ‘Heterochromatinization induced by GAA-repeat hyperexpansion in Friedreich's ataxia can be reduced upon HDAC inhibition by vitamin B3.’ Hum Mol Genet.” copyright (2013). (Chan, Torres et al. 2013)

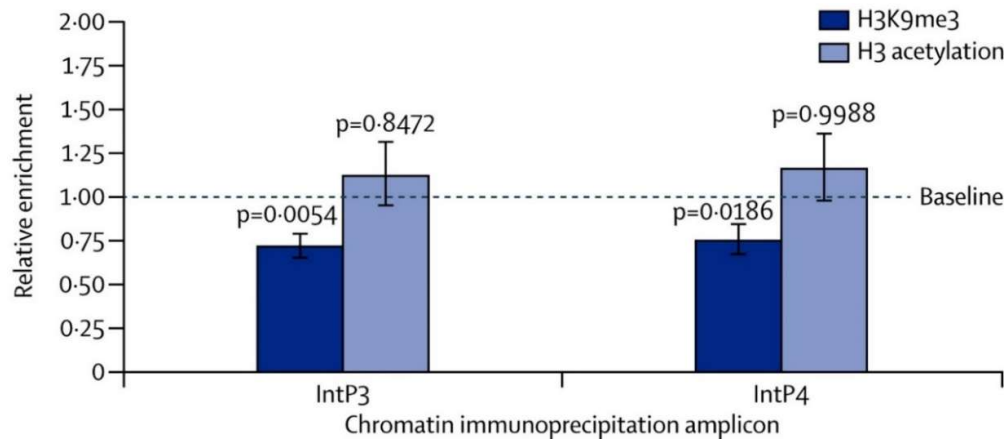


Figure 3.34 Oral intake of nicotinamide reduced the level of H3K9me3 on the *FXN* gene in primary lymphocytes derived from FRDA patients. Nicotinamide treatment of FRDA patients orally was able to increase both *FXN* mRNA and frataxin protein level towards asymptomatic carriers' level (data not shown). This upregulation of *FXN* gene was associated with significant reduction of the H3K9me3 level on the regions flanking GAA repeat expansions of the pathological *FXN* gene relative to untreated control in primary lymphocytes derived from FRDA patients. The position and name of the primer sets used are indicated in Figure 3.32A. There was a collaborative study in the lab together with another PhD student, Theona Natisivili, from design and optimisation of ChIP to performing the definitive experiments. This figure is adapted by permission from Elsevier (under the terms of the Creative Commons Attribution License): The Lancet "Libri, V., C. Yandim, S. Athanasopoulos, N. Loyse, T. Natisvili, **P. P. Law**, P. K. Chan, T. Mohammad, M. Mauri, K. T. Tam, J. Leiper, S. Piper, A. Ramesh, M. H. Parkinson, L. Huson, P. Giunti and R. Festenstein (2014). 'Epigenetic and neurological effects and safety of high-dose nicotinamide in patients with Friedreich's ataxia: an exploratory, open-label, dose-escalation study.' Lancet 384(9942): 504-513.", copyright (2014). (Libri, Yandim et al. 2014)

3.4.1 shRNA-mediated knockdown of HP1 γ does not affect *FXN* expression in Healthy and FRDA fibroblasts

In order to investigate whether HP1 γ mediates silencing of *FXN* gene in FRDA, a knockdown strategy was adopted to deplete HP1 γ in human fibroblast cell lines derived from healthy or FRDA individuals. Expression level of *FXN* mRNA and frataxin protein were subsequently measured and compared. In this experiment, four HP1 γ -targeting shRNA lentiviral constructs were screened (appendix Figure S17) and two of them were employed (shHP1 γ 1 and shHP1 γ 2) to deplete HP1 γ in healthy (GM07048, GM04503) and FRDA (GM04078, GM03665) cell lines with pathologically expanded GAA repeats.

By qRT-PCR, it was confirmed that *FXN* mRNA levels in the FRDA cell lines (GM04078, GM03665) were at levels similar to those found in patients that is significantly lower than healthy control levels. Thus, *FXN* mRNA level of FRDA cell lines GM04078 and GM03665 were 40% and 27% of that of the healthy cell line GM07492 respectively (Figure 3.35A). Frataxin protein levels were also measured in these cell lines using a frataxin Protein Quantity Dipstick Assay Kit (Dipstick Assay). Consistent with the qRT-PCR result, frataxin protein levels in the FRDA cell lines were lower than healthy controls. Thus, FRDA cell lines GM04078 and GM03665 showed frataxin protein levels of about 66% and 25% of healthy control GM07492 (Figure 3.35B).

Upon HP1 γ -targeting lentiviral infection, HP1 γ mRNA and protein levels were significantly reduced in healthy and FRDA cells lines as analysed by qRT-PCR (Figure 3.36) and western blot analysis (Figure 3.37) respectively. Among the two HP1 γ targeting shRNA constructs used in this experiment, construct shHP1 γ 2 showed the most efficient reduction in HP1 γ level, giving 10-23% HP1 γ mRNA of untreated control (UT) and undetectable HP1 γ protein level measured by western blotting (Figure 3.37) in different cell lines whereas construct shHP1 γ 1 showed a relatively mild effect giving 38-79% reduction in HP1 γ mRNA level.

In response to HP1 γ -KD, construct shHP1 γ 1 showed upregulation of *FXN* mRNA of about 1.5 fold in healthy fibroblast GM07492 and GM04503 (Figure 3.38A and

C). Unexpectedly, infection by construct shHP1 γ 1 gave a mild reduction of FXN mRNA level in FRDA cell line GM04078 while that of GM03665 was slightly increased (Figure 3.38B and D). Infection by construct shHP1 γ 2 which gave the greatest HP1 γ reduction did not show increase of FXN mRNA expression of about 1.2 and 2.2 fold respectively in healthy fibroblast GM07492 and GM04503 (Figure 3.38A and C). FXN mRNA level was slightly reduced in FRDA cell line GM04078 (Figure 3.38B). Although construct shHP1 γ 2 led to 2 fold upregulation of FXN mRNA in GM03665, the control vectors with scramble shRNA (Scr) and no insert empty vector (Em) were both able to upregulate FXN mRNA in this cell line (Figure 3.38D) indicating the effect observed may not be due to depletion of HP1 γ level.

Frataxin protein levels were also measured in these cell lines with or without HP1 γ -targeting lentiviral infection by Dipstick Assay. Upon HP1 γ -KD, both construct shHP1 γ 1 and shHP1 γ 2 did not give any statistical significant effect on frataxin protein level in all the cell lines except GM04078 in which infection by construct shHP1 γ 2 caused a mild reduction in frataxin protein level (Figure 3.39B).

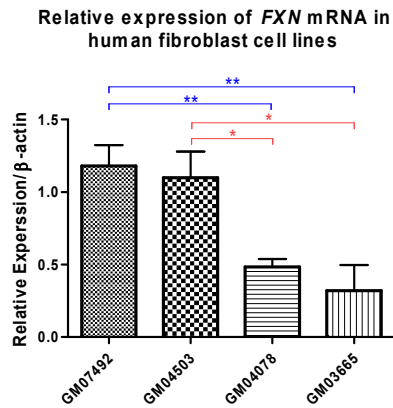
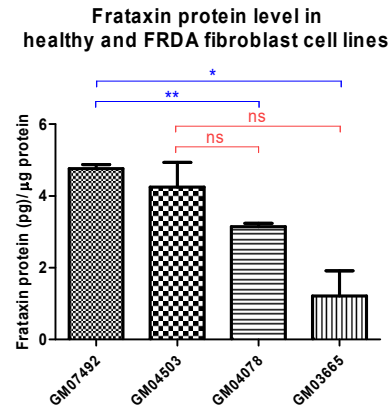
A**B**

Figure 3.35 Relative expression of *FXN* mRNA and protein in healthy and FRDA fibroblasts. Relative expression of *FXN* mRNA (A) and frataxin protein (B) in human fibroblast cells derived from healthy (GM07492, GM04503) or FRDA (GM04078, GM03665) individuals were measured by qRT-PCR and dipstick assay respectively. (A) *FXN* mRNA levels were significantly lower in FRDA cell lines compared to healthy controls. Student's t-test: * $P < 0.05$; ** $P < 0.01$ (compared to GM07492 or GM04503 after being normalised against β -actin expression). Error bars: SEM, $n \geq 3$. (B) Frataxin protein levels are higher in healthy fibroblasts (GM07492, GM04078) comparing to FRDA fibroblasts (GM04078, GM03665). Student's t-test: * $P < 0.05$; ** $P < 0.01$; ns = not significant (compared to GM07492 or GM04503). Error bars: SEM, $n = 2$.

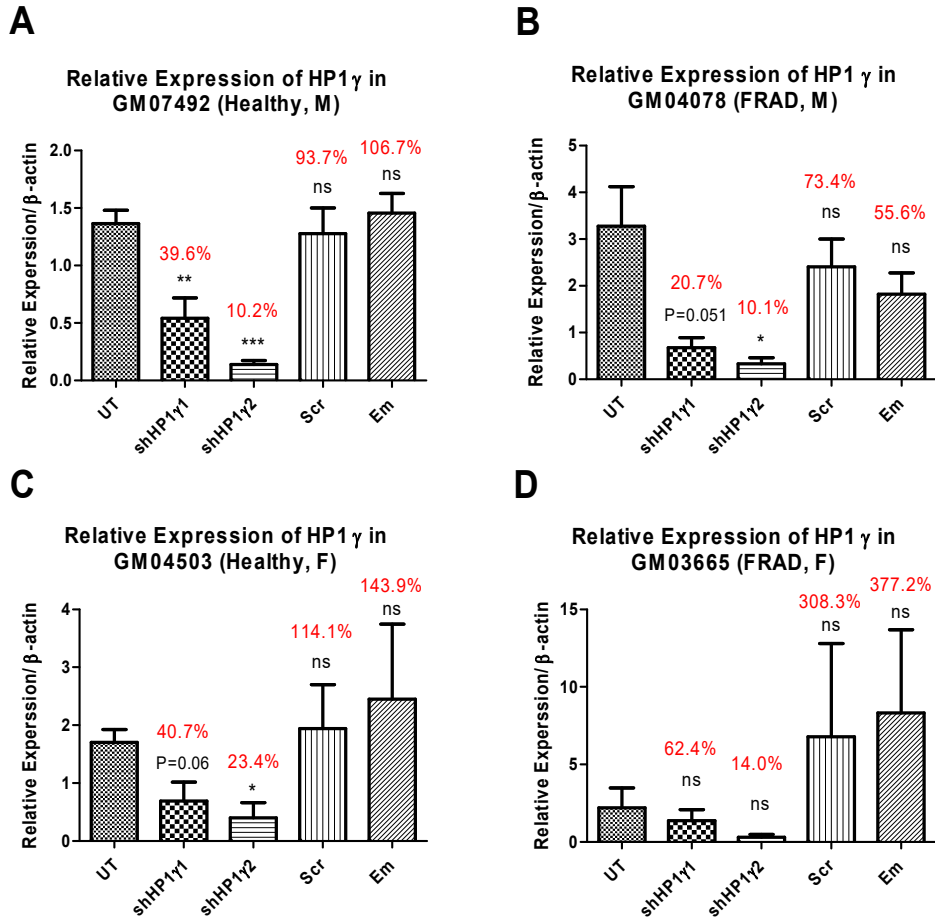


Figure 3.36 Relative expression of HP1γ mRNA in healthy and FRDA cell lines upon shRNA-mediated HP1γ knockdown. HP1γ mRNA levels were efficiently reduced in each fibroblast cell line upon shRNA-mediated knockdown by two different HP1γ-targeting lentiviruses (shHP1γ1 and shHP1γ2). Non-targeting control with scrambled shRNA sequence (Scr) or no shRNA insert (Em) were also included. shHP1γ2 showed a generally better efficiency in depleting HP1γ level giving 10-23% HP1γ mRNA of untreated control (UT) whereas shHP1γ1 showed a relatively mild effect giving 38-79% reduction in HP1γ mRNA level. Levels HP1γ mRNA compared to untreated control are shown in percentage on top of the data bars. Student's t-test: ***P<0.0005; **P<0.01; *P<0.05; ns = not significant (compared to UT after being normalised against β-actin expression). P-values of 04503 and 04078 treated with construct shHP1γ1 were indicated on the graph. Error bars: SEM. n≥3 for GM07492 and GM04078; n≥2 for GM04503 and GM03665.

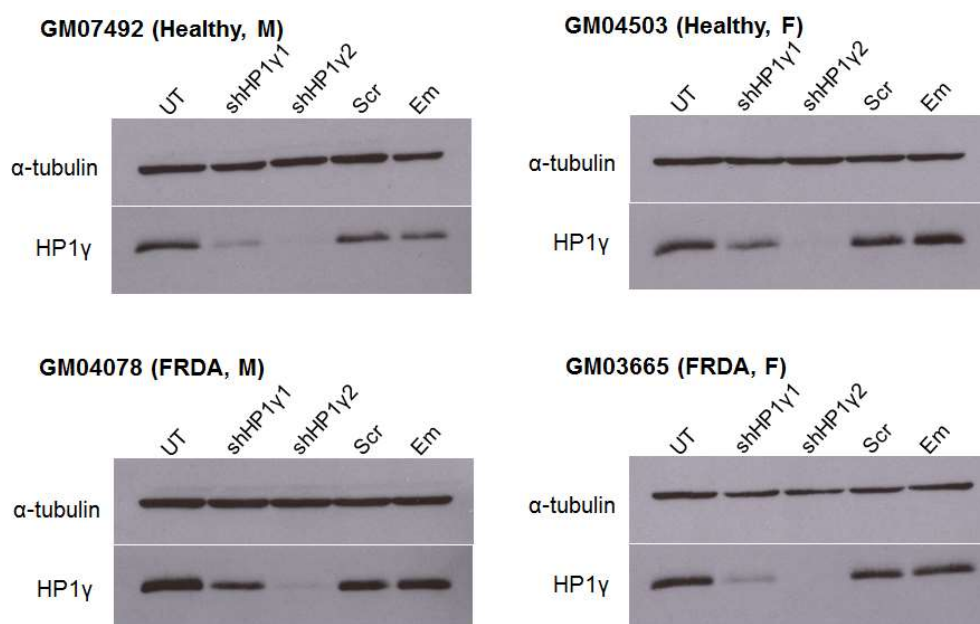


Figure 3.37 Western blot analysis showing HP1 γ protein level in healthy and FRDA fibroblasts upon shRNA-mediated HP1 γ knockdown. Upon shRNA-mediated knockdown, HP1 γ was greatly reduced in cells treated with HP1 γ -targeting lentivirus shHP1 γ 1 while HP1 γ was undetectable in cells treated with lentivirus shHP1 γ 2. Non-targeting control with scrambled shRNA sequence (Scr) or no shRNA insert (Em) showed similar level of HP1 γ comparing to untreated control (UT). Alpha-tubulin (α -tubulin) was used as loading control. Western blots presented here are representative of at least 3 experiments.

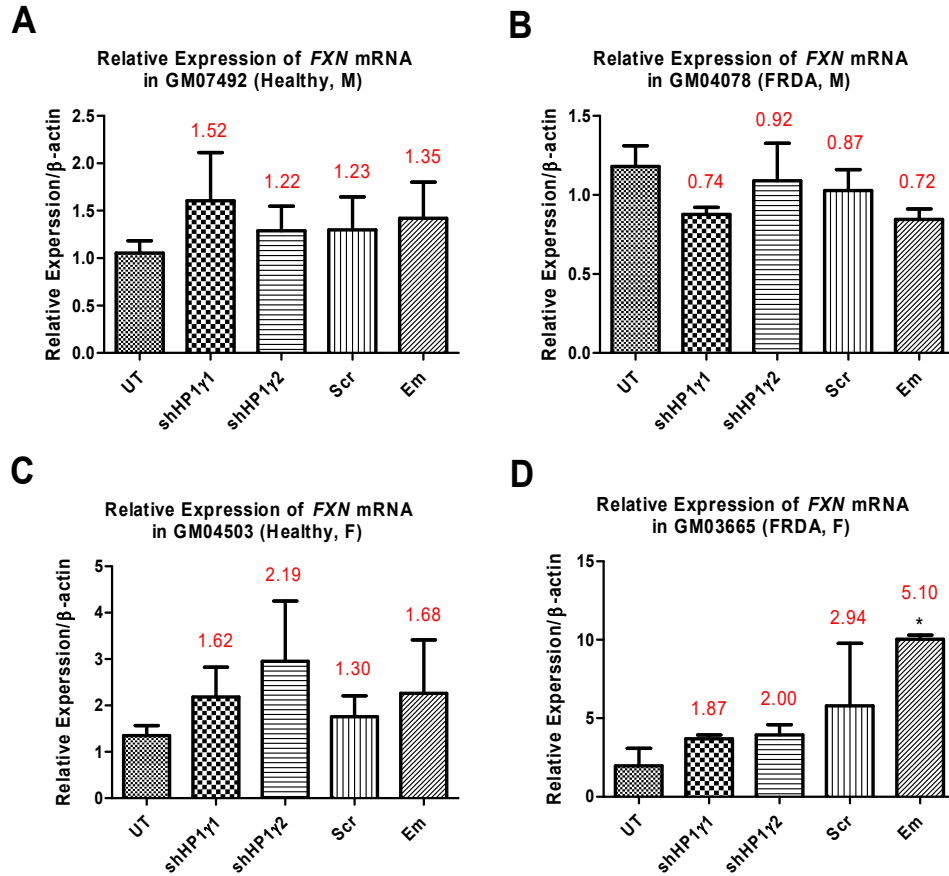


Figure 3.38 Relative expression of *FXN* mRNA in healthy and FRDA fibroblasts upon shRNA-mediated HP1 γ knockdown. Although cells treated with HP1 γ -targeting lentiviruses shHP1 γ 1 and shHP1 γ 2 showed upregulation of *FXN* mRNA comparing to untreated control (UT) in most cases, none of these data are statistically significant. Of note, the control vectors with scramble shRNA (Scr) and no insert (Em) were both be able to upregulate *FXN* mRNA in GM03665 indicating the non-specific effect by shRNA treatment. Fold change of *FXN* mRNA compared to UT are shown on top of the data bars. Student's t-test: * $P < 0.05$; all other data are statistically significant (compared to UT after being normalised against β -actin expression). Error bars: SEM, $n \geq 3$ for GM07492 and GM04078; $n \geq 2$ for GM04503 and GM03665.

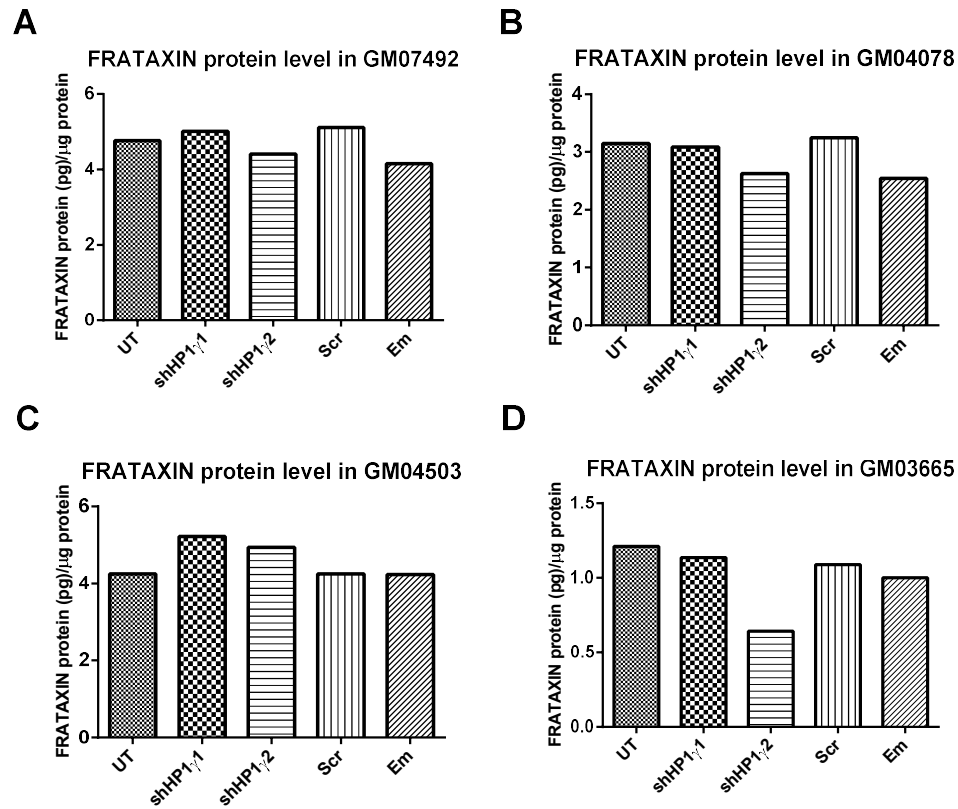


Figure 3.39 Frataxin protein level in healthy and FRDA fibroblasts upon shRNA-mediated HP1 γ knockdown. No significant change in Frataxin protein level was observed in healthy and FRDA fibroblasts upon HP1 γ knockdown except GM04078 treated with lentivirus shHP1 γ 2 which caused a mild reduction in Frataxin protein level. n=2.

3.4.2 Heterozygous knockout of HP1 γ did not affect *FXN* expression in mice cerebella

In order to investigate further the potential effect of HP1 γ on *FXN* gene regulation *in vivo*, double transgenic mice with heterozygous HP1 γ knockout and *hFXN* transgene with pathologically expanded GAA repeats were generated MEFs (see methods and material section 2.3.2.4 for details of the mouse model). Cerebella which are affected in FRDA patients were obtained from these mice and analysed by qRT-PCR. Figure 3.40 shows that the HP1 γ mRNA in the mice with heterozygous HP1 γ knockout (HET) was significantly lower than that of the mice with WT HP1 γ (Figure 3.40A). However, reduced level of HP1 γ did not lead to significant change in *FXN* mRNA level in the cerebella of these animals (Figure 3.40B).

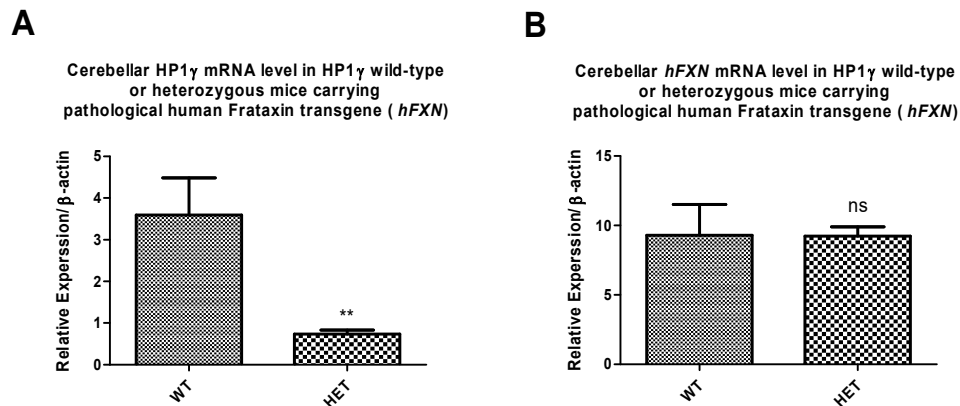


Figure 3.40 Relative expression of HP1 γ and *FXN* mRNA in the cerebella of HP1 γ wild type or heterozygous knockout mice carrying pathological *hFXN* transgene. (A) HP1 γ heterozygous (HET) knockout mice showed significant lower level of HP1 γ mRNA in their cerebella comparing to WT. (B) HP1 γ HET knockout mice showed similar level of *FXN* mRNA comparing to WT. Student's t-test: ** $P < 0.01$; ns = not significant (compared to WT after being normalised against β -actin expression), Error bars: SEM, $n \geq 5$.

3.4.3 Summary of result section 3.4

This section focused on the mechanisms of the silencing of *FXN* gene in FRDA.

The main findings were as follows:

1. Pathological *FXN* gene silencing in FRDA could be reversed by a histone deacetylase inhibitor (nicotinamide) which resulted in a gain in histone H3 and H4 acetylation and a concomitant loss of the classical heterochromatin modifications H3K9me3.
2. It has been shown previously that GAA-repeat induced silencing in mammals can be modulated *in vivo* by changing the dose of the ‘reader’ of H3K9me3 HP1 β , implicating HP1 β as a potential disease modifier. HP1 γ (an isoform of HP1 β) was shown, here and by others, to bind to the *FXN* gene. However, shRNA-mediated knockdown of HP1 γ did not affect *FXN* expression in healthy and FRDA fibroblasts.
3. In order to investigate whether HP1 γ was important for maintaining pathological *FXN* silencing *in vivo*, mouse model which carries pathological *hFXN* transgene was crossed to heterozygous HP1 γ knockout mice and the offspring was analysed. Despite very low levels of HP1 γ , *hFXN* expression in the cerebella of these mice was unaffected. Thus, HP1 γ does not seem to contribute to *FXN* silencing in FRDA.

Chapter 4 - Discussion and Future work

4.1 HP1 γ acts as an activator as well as repressor in the genome of MEFs but the exact mechanisms of regulation remain uncertain

HP1 γ is an isoform of the HP1 family protein and is distinguished by its distinct localisation pattern in both the euchromatic and heterochromatic compartments in interphase nuclei (Horsley, Hutchings et al. 1996, Minc, Allory et al. 1999, Nielsen, Ortiz et al. 1999, Minc, Courvalin et al. 2000). Interestingly, HP1 γ has been implicated in both activation and repression of genes (see introduction section 1.4.2).

In order to gain a better insight into the role of HP1 γ in the regulation of gene expression *in vivo*, genome-wide transcriptional profiling was performed on MEFs with or without HP1 γ . In this experiment, a large number of upregulated (3684 genes) or downregulated (2005 genes) genes were revealed in male samples in response to HP1 γ deletion. A similar result was obtained for female samples although the magnitude of effect was smaller (597 upregulated genes and 148 downregulated genes were revealed) (see section 4.2 for discussion on males and females difference).

This finding is in line with two previously published studies (Smallwood, Hon et al. 2012, Sridharan, Gonzales-Cope et al. 2013), however, a much smaller number of dysregulated genes were reported. Smallwood *et al* revealed 340 upregulated and 367 downregulated genes by at least two fold upon shRNA-mediated HP1 γ knockdown in the human colorectal carcinoma cell line HCT116 (Smallwood, Hon et al. 2012). Although a more stringent threshold in terms of fold change was used in this study (2 fold), than our study (1.5 fold), the number of dysregulated genes was still higher in our study even when the 2 fold threshold was applied to our data. Thus, 2629 upregulated genes and 982 downregulated genes by at least 2 fold (adjusted P-value <0.05) upon HP1 γ knockout. Similar to this study, Sridharan and colleagues showed 352 upregulated and 368 downregulated genes by at least 1.5 fold upon siRNA mediated HP1 γ knockdown in mouse pre-iPSCs (Sridharan,

Gonzales-Cope et al. 2013). Moreover, our study revealed more upregulated genes than downregulated genes in both males and females suggesting HP1 γ might act more often as a silencer than an activator. In contrast, a similar number of up- and downregulated genes were reported by both Smallwood *et al* and Sridharan *et al*.

There are several possible explanations for the difference between our study and the previous studies. Firstly, the genes regulated by HP1 γ and the level of effect may be cell type and species specific. Secondly, the threshold in terms of statistical significance (P-value) that these studies set may not be the same as shown here. Thirdly, to identify genes regulated by HP1 γ , both of these studies employed a knockdown approach where HP1 γ mRNAs were targeted for degradation by shRNA or siRNA for a period of time. This post-transcriptional silencing approach may not provide complete removal of the target mRNA and hence the protein. In our study, we employed a knockout approach where no functional mRNA transcript of HP1 γ was produced. This approach provided cells with complete removal of functional mRNA transcripts and hence protein for the entire life of the cell. These differences in the experimental setups suggest that the expression of some genes may not be affected as the low level of HP1 γ present in the knockdown may still be able to establish and/or maintain their gene expression levels. Their dysregulation might only occur when the HP1 γ level is lower than a certain threshold which would be achieved in the knockout. It is also noteworthy that HCT116 is a male cell line which suggests that the difference in the number of observed dysregulated genes between the study by Smallwood *et al* and ours is unlikely to be due to sex difference.

The underlying mechanisms of how these differentially expressed genes are regulated by HP1 γ directly or indirectly remain unclear. The potential regulatory mechanisms are summarised and discussed here:

4.1.1 Potential direct mechanisms of gene regulation by HP1 γ

Firstly, HP1 γ may recruit other regulatory factors to the target genes that it binds to. For example, HP1 γ has been shown to interact with both initiating and elongating forms of RNAPII (Vakoc, Mandat et al. 2005, Lomberk, Bensi et al. 2006, Mateescu, Bourachot et al. 2008, Smallwood, Hon et al. 2012) and suggested to stabilise ongoing transcription (Reviewed in - Kwon and Workman 2011). Also, HP1 γ was shown to interact with the co-repressor TIF1 β (Brasher, Smith et al. 2000, Lechner, Begg et al. 2000) and the DNA methyltransferase Dnmt1 (Smallwood, Esteve et al. 2007). A list of HP1 γ interacting partners can be found in the introduction section 1.4.2.

Secondly, HP1 γ may also play a role in promoting the formation of local higher order chromatin structures and in turn maintain silencing of the target genes. This idea stems from the finding of a physical interaction between HP1 γ and the H3K9me3 HMTs SUV39H1 (Stewart, Li et al. 2005, Fritsch, Robin et al. 2010) and G9a (Fritsch, Robin et al. 2010). Recruitment of H3K9me3 HMT to the target gene locus might induce further methylation of H3K9 at the locus. These methylated H3K9 residues then provide binding sites for HP1 γ as well as the other HP1 isoforms. Thus, target gene loci with dense H3K9me3 and HP1 protein binding might result in tight crosslinking of nucleosomes, by dimerisation of HP1 proteins, and hence the formation of a condensed chromatin structure (Figure 4.1) (Reviewed in - Hiragami and Festenstein 2005).

Moreover, HP1 γ which has been implicated in regulation of RNA processing (Saint-Andre, Batsche et al. 2011, Ameyar-Zazoua, Rachez et al. 2012, Smallwood, Hon et al. 2012) may maintain gene expression levels by ensuring proper splicing of the mRNA transcripts and hence prevent degradation of mis-spliced transcripts. To address this hypothesis, Saint-Andre *et al* hybridised the total RNA obtained from HeLa cells with HP1 γ knockdown onto exon arrays to test for their exon composition (Saint-Andre, Batsche et al. 2011). The authors reported 10.5% of genes analysed on the arrays showed changes in their exon composition in the absence of HP1 γ . However, only one-third of these genes had disrupted gene expression. More recently, Smallwood *et al* identified 1037 transcripts showing a

marked increase in intronic expression in HCT116 cells upon HP1 γ knockdown (Smallwood, Hon et al. 2012). Significant enrichment of HP1 γ by ChIP has also been shown on genes with increased unspliced transcripts upon knockdown of HP1 γ suggesting a direct effect of HP1 γ . However, only 85 genes out of the 367 downregulated genes in HP1 γ knockdown cells showed increased unspliced transcripts. Taken together, these studies suggested that HP1 γ regulates expression of a subset of genes by maintaining their proper splicing. However, it may also regulate gene expression by alternative mechanisms.

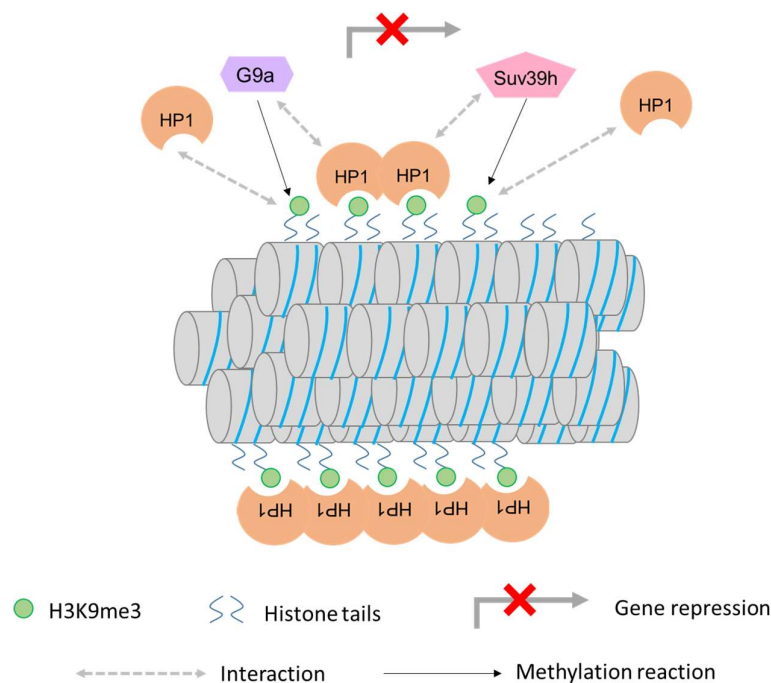


Figure 4.1 Schematic diagram demonstrating the potential mechanism of HP1 γ in promoting heterochromatin formation. HP1 γ can potentially affect gene expression directly by promoting the local higher order chromatin structure formation. HP1 γ which bound to H3K9me3 on the nucleosome can recruit the H3K9me3 HMTs SUV39H1 or G9a to the target locus and hence promote further methylation of the locus. Methylated H3K9 residues then provide binding sites for HP1 γ as well as other HP1 isoforms. HP1 proteins can dimerise to form homo- or heterodimers and hence crosslink the nucleosomes into condensed chromatin structure.

4.1.2 Potential indirect mechanisms of gene regulation by HP1 γ

Firstly, HP1 γ may indirectly affect gene expression by regulating genes (e.g. genes encoding transcription regulators) which in turn regulate other genes or groups of genes.

Secondly, HP1 γ may affect gene expression by regulating nearby regions of heterochromatin including annotated repeats or pericentric repeats. As mentioned earlier, HP1 γ can potentially establish and/or maintain heterochromatin by its interaction with the H3K9me3 HMTs. HP1 γ , hence, may mediate spreading of heterochromatin and cause silencing of genes in nearby regions.

Thirdly, HP1 γ may also affect gene expression by indirectly regulating mRNA splicing. This hypothesis is supported by the GO term analysis presented here (Table S2 in appendix) as well as the statistical overrepresentation test showing enrichment of functional groups involved in RNA processing associated with the downregulated genes upon HP1 γ knockout (Table S6 in appendix) (see also result section 3.1.4 and 3.1.5). This result suggested that HP1 γ might regulate expression of components of the splicing machinery and hence indirectly affect the proper processing of mRNA transcripts. Improperly spliced transcripts may then be degraded.

4.2 HP1 γ is implicated in regulation of sexually dimorphic expression of genes

Males and females are different in physiology, behaviour as well as susceptibility to many diseases (Reviewed in - Wijchers and Festenstein 2011). As mentioned in the introduction (section 1.7), sexual differences are determined by three main factors: activational effects and organisational effect of gonadal steroid hormone and sex chromosomes (Reviewed in - Arnold 2009).

Previously, a study by our group showed a significant number of autosomal genes that are epigenetically regulated by sex chromosome complement (XY or XX) rather than phenotypic sex using a 'sex-reversal' mouse model in which the mice were either deficient or transgenic for the sex determining *Sry* gene (Wijchers, Yandim et al. 2010). Thus, XX mice carrying the *Sry* transgene become phenotypic males and XY mice with the *Sry* gene deleted become phenotypic females. Interestingly, XY cells displayed significantly more heterochromatin-mediated silencing in a mammalian model for PEV than XX cells irrespective of phenotypic sex. Moreover, a greater proportion of HP1 β sensitive genes (identified by microarray analysis comparing HP1 β overexpressing cells compared with WT) were found in the XY cells suggesting a role for HP1 β in regulating sexual dimorphic gene expression that is dependent on sex chromosome complement rather than gender (Wijchers, Yandim et al. 2010).

Interestingly, it was shown in this thesis by RNA-seq analysis that there is a much higher number of dysregulated genes in male MEFs (total of 5689 genes) compared with female MEFs (total of 745 genes) in response to the removal of the HP1 isoform HP1 γ . Some of these dysregulated genes are commonly regulated by both males and females but genes regulated solely by either males or females were also identified. However, while Wijchers *et al* showed that PEV is sexual dimorphic (Wijchers, Yandim et al. 2010) and the higher expression of Mj-sat transcripts were revealed in the four core genotype mice in female thymus versus male (Marta Mauri MSc thesis, Imperial College London), it is shown here that pericentric Mj-sat expression is not dimorphic in its expression but dependent on HP1 γ in both male and female MEFs to the same extent. In the investigation of the physiological

functions influenced by HP1 γ deficiency, we found by GO term analysis that there are a higher number of dysregulated functional gene groups associated with males rather than females in response to HP1 γ knockout. This is consistent with there being a greater number of genes affected in males than females. Also, distinct functional groups were found to be associated with males and females in response to HP1 γ knockout. These findings suggest that males have a higher dependency on HP1 γ for maintaining their normal gene expression profile. Taken together, these results may reflect sexual dimorphism in both gene expression and response to HP1 γ deficiency. HP1 γ may compensate for an underlying difference in gene expression between sexes that is due to sex chromosome complement.

4.2.1 The effect of HP1 γ in regulating the 130 genes that show sexually dimorphic gene expression in WT MEFs

To address this hypothesis, we tested the genes that were differentially expressed in males and females for their sensitivity to HP1 γ deficiency. Among the 130 sexually dimorphic genes, 85 genes have higher expression in WT females (normally repressed in males) and 45 genes have higher expression in WT males (normally repressed in females). 97 out of the 130 sexually dimorphic genes were found to be dysregulated in males while only 29 genes were found to be dysregulated in females. These data indicated that genes showing sexually dimorphic expression are enriched in HP1 γ sensitive genes. Moreover, a greater proportion of HP1 γ sensitive genes were found in the males than females. Regarding the repressive role of HP1 γ , compared to the percentage of the total number of upregulated genes (3684/23425, 15.7% for males and 597/23425, 2.5% in females) the percentage of the 130 genes, which showed sexually dimorphic expression, that were upregulated in response to HP1 γ knockout was significantly skewed in both males (72/130, 55.4%) and female (18/130, 13.8%). Taken together, these findings suggest that males have a higher dependency on HP1 γ for maintaining the expression level of these sexual dimorphic genes which are set at a lower level in males than females. Also, the higher number of upregulated genes revealed in both males and female suggests that HP1 γ acts more often as a silencer to repress the expression of these sexually dimorphic genes.

4.2.2 The effect of HP1 γ deficiency on the genes repressed more in WT males than females

Indeed, in the investigation into the role of HP1 γ in maintaining gender-specific expression of the sexually dimorphic genes, we found that removal of HP1 γ caused derepression of almost all significantly changed genes (69/70 significantly changed genes upregulated in response to HP1 γ knockout in males) that were normally repressed in WT males (85 genes). Notably, little effect of HP1 γ deficiency on the 130 genes with sexually dimorphic expression was shown in females where only a small number of them were significantly changed (16/85 genes) and both up- (6 genes) and downregulation (10 genes) of genes were observed. These data indicated that HP1 γ is playing a role in repressing these 85 genes in WT males but not in WT females.

4.2.3 The effect of HP1 γ deficiency on the genes that are activated more in WT males than females

45 of the 130 genes showing sexually dimorphic expression were higher in WT males (45 genes) than WT females. Removal of HP1 γ caused repression of 24 of these genes, whilst only 3 were upregulated and the remainder unaffected. In females only 13 of the 45 of these sexually dimorphic genes were affected by HP1 γ deficiency. Interestingly, almost all of those significantly changed (12/13 significantly changed genes) were found to be upregulated in response to HP1 γ knockout in females. These data indicate that HP1 γ is playing an opposite role in regulating these 45 genes in WT males and females. These opposite effects suggest that different mechanisms are operating in males versus females which would also be secondary to the possible sex chromosome complement effects described above.

Overall, these results suggest that HP1 γ is playing a role in maintaining sexual differences by maintaining the sex-specific difference in expression of these sexually dimorphic genes. Moreover, these results also highlighted two interesting features of HP1 γ in gene regulation: (1) HP1 γ appears to have a relatively clear-cut

male-specific repressive role on a subset of the sexually dimorphic genes; (2) HP1 γ appears to have opposite roles in males versus females on those genes that are normally expressed at a higher level in males than females.

As the study was performed in MEFs derived from E13.5 embryos the result implies that these sex-specific effects are likely to be due to sex chromosome complement rather than sex hormones as the latter are only produced in significant quantities much later in development. There are several possible ways in which the XY and XX chromosome complements might be responsible for the effects described here.

Firstly, the Y chromosome carries male specific genes several of which are known chromatin modifiers for example *Kdm5d* and *Uty* which were found to be expressed in the MEFs analysed here. *Kdm5d* is a histone demethylase which targets the active modification H3K4me3 and would therefore be a good candidate for participating in gene repression. Indeed, knockdown of this gene in MEFs has been shown in our lab to upregulate Mj-sat repeats (Marta Mauri and Raquel Silva MSc theses, Imperial College London). It is therefore possible that HP1 γ interacts in some way with either this gene or other Y-specific genes to silence the 85 genes here shown to be repressed in WT males compared with females. Previous attempts to knockout Y chromosome genes in ES cells proved virtually impossible but now with the advent of clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 technology it is possible (Wang, Yang et al. 2013). Hence, the genetic interaction of these Y-specific genes with HP1 γ could be investigated in the future work.

Secondly, the phenomenon of genes escaping X-inactivation in XX cells (Carrel and Willard 2005) might also be responsible for protecting females from the repressive effect of HP1 γ on the 85 genes that are normally expressed at a lower level in males compared with females.

Thirdly, another possibility is that the inactive X-chromosome in females might be acting as a heterochromatic 'sink' thereby sequestering silencing factors away from the euchromatic regions (Chadwick and Willard 2003) allowing higher expression of the 85 genes that show higher expression in WT females compared to males. The simplest model would be that HP1 γ would be sequestered to the inactive X in

females in line with the finding that H3K9me3 decorates the inactive X-chromosome (Heard, Rougeulle et al. 2001). However, it was reported in the same paper that HP1 isoforms are not enriched on the inactive X although the data were not shown and the isoforms not specified (Heard, Rougeulle et al. 2001).

Taken altogether, the results obtained implicate HP1 γ in the regulation of sexually dimorphic expression of genes and are consistent with previous studies from our lab which demonstrated that heterochromatin-mediated silencing was subject to sex-chromosome complement effect. (Wijchers, Yandim et al. 2010).

4.3 HP1 γ is likely to be regulating the common enhancer rather than imprinting of the *Igf2/H19* locus

Genomic imprinting is an epigenetic process that maintains allelic expression of genes according to their parental origin (Reviewed in - Morison, Ramsay et al. 2005). (Reviewed in - Kelsey and Feil 2013). A lot of these imprinted genes are important for growth and development as well as other processes such as energy homeostasis, brain function and behaviour (Reviewed in - Nordin, Bergman et al. 2014). Dysregulation of imprinted genes has also been found to be associated with various diseases (Reviewed in - Morison, Ramsay et al. 2005).

In our investigation into the loss of function of HP1 γ , significant enrichment of dysregulated imprinted genes were revealed in which ~40% of the total number of imprinted genes were up- or downregulated by at least 1.5 fold in response to HP1 γ deletion. In this analysis, dramatic dysregulation of the *Igf2* and *H19* genes were observed in which both of these genes were downregulated by 12.9 and 13.3 fold respectively in response to HP1 γ knockout.

There are several possible mechanisms by which HP1 γ might regulate the *Igf2/H19* locus. Firstly, a previous study showed concentration of HP1 γ at the 3' end of active genes (Vakoc, Mandat et al. 2005, Smallwood, Hon et al. 2012) and its interaction with both serine-2 and serine-5 phosphorylated RNAPII (Vakoc, Mandat et al. 2005), HP1 γ might therefore stabilise the transcripts of *Igf2* and *H19* and hence maintain their expression level. Also, HP1 γ which has been implicated in the regulation of RNA processing (Saint-Andre, Batsche et al. 2011, Ameyar-Zazoua, Rachez et al. 2012, Smallwood, Hon et al. 2012) may maintain the expression levels of the *Igf2* and *H19* gene by ensuring proper RNA splicing to produce correct mRNA transcripts and hence preventing degradation of mis-spliced transcripts by nonsense-mediated decay. HP1 γ could also possibly indirectly regulate the expression of these genes by regulating the expression of the upstream activator of the *Igf2* and *H19* gene.

Our data do not provide strong evidence which favour any of these possible mechanisms. However, as mentioned in introduction section 1.5, *Igf2* and *H19*

genes which are located at the same locus are reciprocally expressed from both alleles according to the parental inheritance and their expression is regulated by a common enhancer. The fact that both of these genes were downregulated by a similar magnitude (fold change) raised the possibility that HP1 γ might be regulating the enhancer function which has not been suggested previously.

In this case, HP1 γ might regulate the *Igf2/H19* locus by promoting the promoter/enhancer communication. It is tempting to speculate that this mechanism might involve direct binding of HP1 γ to the promoters (*Igf2* promoter on the paternal allele and *H19* promoter on the maternal allele) and/or the common enhancers on both alleles. Binding of HP1 γ to both promoter and enhancer may allow its homodimerisation and hence the promoter and enhancer might be brought in close proximity for promotion of gene activation. HP1 γ may also bind either promoter or enhancer and interact with other promoter/enhancer specific factors to assist the promoter/enhancer interaction. Of course another possibility is that HP1 γ deficiency might affect such looping by an indirect, as yet undetermined mechanism. Association of HP1 γ to the gene promoter has been shown by ChIP-seq analysis in MEFs induced to become pre-iPSCs (Sridharan, Gonzales-Cope et al. 2013). Moreover, association of HP1 γ with an enhancer has been shown previously in regulatory T (Treg) cells in which HP1 γ , together with H3K9me3 and SUV39H associated with the Treg-specific demethylation region (TSDR) enhancer element of the *FOXP3* gene. However, association of HP1 γ , H3K9me3 and SUV39H on TSDR functioned to promote silencing of the *FOXP3* gene (Sarmiento, Svingen et al. 2015) rather than playing an activation role as observed in the case of *Igf2/H19* locus.

To investigate whether HP1 γ is playing a role in promoter/enhancer interaction, the MEF-specific enhancer element for the *Igf2/H19* locus has to be identified. This can be facilitated by aligning the profile of H3K27ac and H3K4me which are the histone modifications that mark enhancer elements (Barski, Cuddapah et al. 2007, Creighton, Cheng et al. 2010). The level of HP1 γ enrichment on the enhancer element can hence be determined by ChIP followed by qRT-PCR or high throughput sequencing. Moreover, to determine whether promoter/enhancer interaction is compromised in the absence of HP1 γ , chromosome conformation capture (3C)

would allow determination of interaction frequency between selected genomic regions in cell populations (de Wit and de Laat 2012) can be performed in HP1 γ WT and HOM MEFs. These experiments can also extend our current study further to see whether HP1 γ is an enhancer regulatory factor which is currently largely unknown. Taken together, these findings bring to light a potential role for HP1 γ in gene regulation by regulating enhancer activity.

4.4 HP1 γ is implicated in silencing of the pericentric Mj-sat expression

Pericentric heterochromatin which is adjacent to the centromere of a mouse chromosome is essential for proper chromatin segregation in mitosis as well as maintenance of genome integrity (Reviewed in - Allshire and Karpen 2008, Saksouk, Simboeck et al. 2015). Pericentric heterochromatin is composed predominately of the AT-rich major satellite (Mj-sat) repeat units of up to $\geq 10,000$ copies which contribute to $\sim 3\%$ of the genome (Mouse Genome Sequencing, Waterston et al. 2002).

In *S. pombe*, transcripts derived from pericentric heterochromatin is involved in establishment of heterochromatin formation by an RNA interference (RNAi)-mediated mechanism (Reviewed in - Grewal and Jia 2007). While RNAi machinery can also be found in mammals, relatively little evidence suggest its involvement in establishment of heterochromatin (Reviewed in - Saksouk, Simboeck et al. 2015).

However, various studies suggest that non-coding RNA derived from pericentric heterochromatin potentially has important roles in mammalian cells. Firstly, expression of heterochromatic transcripts are tightly coupled to the cell cycle (Lu and Gilbert 2007) and synthesised into double-stranded RNA (dsRNA) by bidirectional transcription (Martens, O'Sullivan et al. 2005). Secondly, RNA derived from pericentric heterochromatin was implicated in heterochromatin formation during early mouse development (Probst, Okamoto et al. 2010, Santenard, Ziegler-Birling et al. 2010). Also, it has been shown that accumulation of H3K9me3 and HP1 proteins to heterochromatic regions was compromised upon RNase A treatment suggesting the role of an RNA component in maintaining the structure of heterochromatin (Maison, Bailly et al. 2002). Moreover, expression of pericentric transcripts was detected in some human cancers (Eymery, Horard et al. 2009, Ting, Lipson et al. 2011). Hence, expression of pericentric transcripts has been considered to be a characteristic of genome instability and loss of integrity of heterochromatin (Eymery, Horard et al. 2009, Wang, Zhou et al. 2013) and thus a likely prerequisite for oncogenesis (Eymery, Callanan et al. 2009, Eymery, Horard et al. 2009). Controlling expression of pericentric transcripts in mammalian cells therefore

appears key for maintaining genome stability. However, the exact mechanism by which the expression of these transcripts are regulated has not been fully elucidated.

HP1 γ of the HP1 protein family has been implicated predominantly in regulating euchromatic genes rather than heterochromatin due to its nuclear distribution in both euchromatic and heterochromatic compartment in interphase nuclei (Horsley, Hutchings et al. 1996, Minc, Allory et al. 1999, Nielsen, Ortiz et al. 1999, Minc, Courvalin et al. 2000). Also, recent studies revealed a positive correlation between level of enrichment of HP1 γ and gene activity (Smallwood, Hon et al. 2012, Sridharan, Gonzales-Cope et al. 2013). Moreover, localisation of HP1 γ to the heterochromatic region has been reported to be dependent on HP1 α and HP1 β (Dialynas, Terjung et al. 2007, Mosch, Franz et al. 2011). These results led to the notion that HP1 γ is predominately associated with euchromatic gene regulation.

In this thesis, the function of HP1 γ in pericentric heterochromatin was investigated. Interestingly, knockout of HP1 γ in both male and female MEFs showed an average of 5 fold increase in Mj-sat expression as detected by qRT-PCR. Similar results were also observed here in the human cell line, HeLa with knockdown of HP1 γ . These data suggest that HP1 γ is a silencer of the pericentric Mj-sat transcripts and potentially plays an important role in regulating pericentric heterochromatin silencing.

A variety of protein factors have also been implicated in maintaining the silencing of the pericentric Mj-sat transcripts expression in mammals. Interestingly, many of these factors are interacting partners of HP1 γ in mammals. These factors include the chromatin modifiers SUV39H1 and SUV39H2 (Lehnertz, Ueda et al. 2003, Martens, O'Sullivan et al. 2005, Mosch, Franz et al. 2011) and KDM2A (Frescas, Guardavaccaro et al. 2008), as well as the transcription factors PAX3 (Bulut-Karslioglu, Perrera et al. 2012) and BRCA1 (Zhu, Pao et al. 2011). For references for their interaction with HP1 γ see Table 1.2 in introduction section 1.4.2. It is also noteworthy that Mj-sat derepression has also been reported in cells upon removal of both HP1 α and HP1 β (Mosch, Franz et al. 2011). Discussion regarding the relationship between the three HP1 isoforms can be found in section 4.5. Removal or reduction of these factors in various mammalian model systems were found not

only to be associated with derepression of Mj-sat expression but also various epigenetic changes in the pericentric heterochromatin such as reduction of H3K9me3 (for PAX3, SUV39H), reduction in H4K20me3 (for PAX3), reduction of DNA methylation (for SUV39H1), reduction of mono-ubiquitinated H2A (for BRCA1) and an increase in H3K36me2 (for KDM2A).

In our study, immunofluorescence microscopy showed that deletion of HP1 γ did not lead to a pronounced change in pericentric heterochromatin as indicated by DAPI staining. Also, localisation of H3K9me3 in DAPI-dense regions which contains the pericentric heterochromatin was not significantly changed. Enrichment levels of H3K9me3 at the pericentric regions were also analysed by ChIP-qRT-PCR on Mj-sat regions and no significant changes were observed in response to HP1 γ depletion. These results are rather surprising given that HP1 γ can recruit SUV39H1 (Stewart, Li et al. 2005, Fritsch, Robin et al. 2010) which establishes and/or maintains H3K9me3 at pericentromeres (Lehnertz, Ueda et al. 2003). Nonetheless, a recent study showed that HP1 proteins are dispensable for the establishment of SUV39H1-mediated H3K9me3 accumulation at the pericentromeric region (Muramatsu, Singh et al. 2013). Moreover, similar results have been shown in cells with double knockout of HP1 α and HP1 β in which upregulation of Mj-sat transcripts were not associated with H3K9me3 changes as analysed by immunofluorescence microscopy (Dialynas, Terjung et al. 2007, Mosch, Franz et al. 2011). Our results suggest that large scale loss of H3K9me3 is unlikely to be responsible for the derepression of heterochromatin transcripts observed in HP1 γ HOM MEFs. Other silencing marks such as H4K20me3 or DNA methylation may be in play. Alternatively, HP1 γ may mediate pericentric heterochromatin silencing by inhibiting active modifications such as histone acetylation on these regions. As mentioned above, there are estimated to be $\geq 10,000$ copies of Mj-sat repeat units in the genome. It is also possible that if derepression only occurs in a small subset of Mj-sat repeats, associated changes in H3K9me3 may not be detectable due to the sensitivity of the methodology used.

Given that HP1 γ interacts with factors involved in silencing of pericentric heterochromatin and its deficiency in male cells results in dysregulation of ~ 5000 genes, it seems likely there might be some commonality between the silencing

mechanisms in these cases. Thus, mRNA expression changes of these factors upon HP1 γ knockout were checked in our RNA-seq data (Table 4.1). Our data showed that mRNA expression of all these factors were not statistically significantly changed in female MEFs. In males, expression of these factors were only modestly downregulated (~1.2-2.4 fold) in response to HP1 γ knockout except PAX3 which upregulated by ~5 fold. As we observed similar magnitude of upregulation of Mj-sat in both males and females (Figure 3.14 in section 3.2.1), these data suggest that HP1 γ is unlikely to be derepressing Mj-sat expression by greatly reducing the expression level of these factors. However, we cannot rule out the possibility that there are other as yet unknown heterochromatin regulatory factors whose expression is regulated by HP1 γ .

Gene	Fold change in response to HP1 γ knockout (Male)	Adjusted P-value	Fold change in response to HP1 γ knockout (female)	Adjusted P-value
<i>Brca1</i>	2.37 (down)	6.21E-13	1.08 (down)	0.80
<i>Kdm2a</i>	1.15 (down)	6.39E-05	1.12 (down)	0.48
<i>Pax3</i>	4.97 (up)	1.77E-06	3.09 (up)	0.16
<i>Suv39h1</i>	1.57 (down)	8.50E-08	1.04 (up)	0.79
<i>Suv39h2</i>	2.1 (down)	1.22E-09	1.00	0.99

Table 4.1 Table summarises the fold change in mRNA expression level of factors which have been implication in pericentric heterochromatin silencing in response to HP1 γ knockout in MEFs.

Interestingly, it has been shown that knockout of BRCA1 led to reduction of the protein level of all the HP1 isoforms (Zhu, Pao et al. 2011) and knockdown of KDM2A caused HP1 γ delocalisation from heterochromatic regions (Frescas, Guardavaccaro et al. 2008). It is also noteworthy that mitotic defects were found to be associated with depletion of both BRCA1 and KDM2A (Frescas, Guardavaccaro et al. 2008, Zhu, Pao et al. 2011) which has also been reported in various models with HP1 γ deficiency (Obuse, Iwasaki et al. 2004, Serrano, Rodriguez-Corsino et al. 2009, Shimura, Toyoda et al. 2011, Grzenda, Leonard et al. 2013). These results suggest that HP1 γ may be in the same regulatory pathway with these proteins, possibly acting as a downstream factor. To address this hypothesis, the epigenetic

changes associated with BRCA1 and KDM2A deficiency i.e. the mono-ubiquitinated H2A and H3K36me2 can be tested in our model.

4.5 Increased proportion of cells with diffuse staining of HP1 β in absence of HP1 γ

In result section 3.2.2, immunofluorescence microscopy revealed two different distribution patterns of HP1 β in WT MEF nuclei (the pan-nuclear distribution and the concentration within DAPI-dense regions) which has also been described previously although this was not quantified (Dialynas, Makatsori et al. 2006, Dialynas, Terjung et al. 2007). Our experiment also showed that the proportion of cells showing diffuse pan-nuclear staining of HP1 β was increased upon HP1 γ depletion. However, removal of HP1 γ did not affect HP1 α distribution in the nucleus. And this effect is not a consequence of changes in overall expression level of the HP1 β estimated by qRT-PCR upon HP1 γ depletion.

Three explanations may be able to account for this observation. Firstly, it has been shown previously in HeLa cells that the nuclear distribution of HP1 β incorporation is cell cycle-dependent where it stably incorporates in heterochromatic domains in late S-phase and at the end of mitosis (Minc, Allory et al. 1999, Dialynas, Makatsori et al. 2006). Although a previous experiment on a different genetic background revealed no cell cycle disruption in MEFs upon HP1 γ depletion (Abe, Naruse et al. 2011), our GO analysis in male MEFs as well as the statistical overrepresentation test suggested that HP1 γ is implicated in cell cycle regulation. And its implication in regulating different stages of the cell cycle has also been suggested by several previous studies in a number of mammalian models e.g. reduction in cell growth (Caillier, Thenot et al. 2010) and mitotic defects (Serrano, Rodriguez-Corsino et al. 2009, Shimura, Toyoda et al. 2011) upon HP1 γ depletion. In the light of the GO analysis presented here and my anecdotal observation that the male MEFs might be proliferating at a slower rate than WT, we now need to take this further and investigate in more detail whether HP1 γ might have a role in maintaining normal cycling of cells in a gender specific manner. Female MEFs have very recently been investigated in this respect and found to have same cell cycle profiles and proliferation rate (using PI staining) when comparing WT MEFs with HP1 γ deficient MEFs (Lakshmi Devi Regnier Cadavieco MSc thesis, Imperial College London).

Secondly, given that HP1 β and HP1 γ are two structurally and biochemically similar proteins of the same protein family (Jones, Cowell et al. 2000), another possibility is that HP1 β may bind to sites previously occupied by HP1 γ thereby potentially compensating for the loss of HP1 γ and leading to re-distribution of HP1 β to euchromatic regions that are normally occupied by HP1 γ . To address this hypothesis, ChIP-seq can be employed to reveal the global binding pattern of HP1 β WT and HP1 γ knockout MEFs. ChIP with antibodies against HP1 β has proved problematic in several laboratories making this experiment a technical challenge. The Festenstein lab has been developing transgenic mice carrying HP1 β fused to a biotin tag to overcome this technical hurdle (Dr. Patrick Wijchers, personal communication).

Thirdly, HP1 proteins are known to form homodimers or heterodimers. Interaction between HP1 isoforms has previously been shown by an *in vitro* assay and pull-down assay using tag fusion proteins (Nielsen, Oulad-Abdelghani et al. 2001). Their interaction has also been shown by using mass spectrometry methodologies in a human cell line (Rosnoblet, Vandamme et al. 2011). Their interaction suggests the possibility that HP1 γ may recruit HP1 β by direct interaction to the heterochromatic regions. Loss of HP1 γ may hence affect the localisation of HP1 β to densely stained heterochromatic regions and lead to its pan-nuclear localisation. Intriguingly, Dialynas *et al* and Mosch *et al* showed in 2007 and 2011 that there was no detectable enrichment of HP1 γ at DAPI-dense regions in MEFs with double knockout of HP1 α and HP1 β (Dialynas, Terjung et al. 2007, Mosch, Franz et al. 2011). These authors hence suggested that HP1 α and HP1 β are necessary for HP1 γ localisation to pericentromeric heterochromatin. Taken altogether, these data suggest the hypothesis that various HP1 homo- or heterodimers that are composed of different combinations of HP1 proteins may contribute to distinct functions in the genome and raises the possibility that specific HP1 dimers are crucial for specific physiological processes, a question which remains as yet unanswered.

4.6 Investigation into the context dependent regulation of gene expression by HP1 γ *in vivo*

This thesis, as well as various previous studies, implicated HP1 γ in regulation of both gene activation and repression (see introduction section 1.4.2 and result section 3.1.2). This interesting feature of HP1 γ being able to regulate both gene activation and repression in the genome prompted us to ask how the active or repressive role of HP1 γ is determined and its nature and mechanisms.

We hypothesised that the mode of HP1 γ regulation is dependent on the ‘context’ i.e. which regulatory factors (including specific epigenetic modifications) it interacts with in different nuclear environments (e.g. euchromatic or heterochromatic).

Indeed, results presented by a number of studies are pointing towards this idea. For example, the study by Smallwood and colleagues in 2007 where the functional cooperation between HP1 proteins and the DNA methyltransferase DNMT1 was investigated. In this study, all of the mammalian HP1 isoforms (HP1 α , HP1 β and HP1 γ) were shown to associate with and be able to stimulate the activity of DNMT1 *in vitro*. They have also shown *in vivo* by use of a reporter system that the three HP1 proteins were capable of inducing eight to thirteen fold repression of the responsive reporter gene by inducing DNMT1-mediated DNA methylation of the reporter gene. These results suggested that HP1 γ , similar to HP1 α or HP1 β is able to repress gene expression in collaboration with DNMT1 (Smallwood, Esteve et al. 2007). However, when investigating this cooperation *in vivo*, they found that HP1 γ was associated with the active promoter of the endogenous gene *Survivin*. Interestingly, upon induced silencing of *Survivin*, the *Survivin* promoter lost enrichment of HP1 γ binding and gained enrichment of HP1 α and HP1 β together with DNMT1, G9a and H3K9me2. In cells in which DNMT1 was deleted, induced silencing of *Survivin* failed but enrichment of HP1 γ remained. Failure of repression was also correlated with loss of enrichment for HP1 α , HP1 β , DNMT1, G9a and H3K9me2 at the *Survivin* promoter. Similar results were also presented for *cdc2* and *cdc25* genes in the wild type situation (Smallwood, Esteve et al. 2007). Taken together, these experiments suggest that whether HP1 γ is an activator or repressor

is dependent on the genomic context. Yet, the exact nature of what defines the response to HP1 γ is largely unknown.

Another example includes a recent study which implicated all three mammalian HP1 isoforms in regulating expression of alternative exons. Interestingly, HP1 isoforms can act to either promote or inhibit exon inclusion alternative exons. Further study into the role of HP1 β in alternative splicing in relation to DNA methylation revealed the context dependent regulation of alternative exon expression by HP1 β : positive regulation of exon inclusion by HP1 β is correlated with high level of both DNA methylation and HP1 β in the body of alternative exons whereas negative regulation of exon inclusion by HP1 β is correlated with lower levels of DNA methylation in the body of exon and binding of HP1 β in upstream intron (Yearim, Gelfman et al. 2015). This study again supports the hypothesis that the function of HP1 is dependent on the ‘context’.

We hypothesise that histone variant H3.3 is one of the potential determinants of the function of HP1 γ suggested by its specific interaction with HP1 γ but not HP1 α and β as shown here and by others (Loyola, Bonaldi et al. 2006, Kim, Heo et al. 2011). Intriguingly, binding of both H3.3 and HP1 γ to the promoters of the HSP70 genes was found to be interdependent in which knockdown of HP1 γ was found to cause a reduction of H3.3 at the promoters of HSP70 genes and vice versa (Kim, Heo et al. 2011). Binding of both H3.3 and HSP70 is also correlated with the expression level of HSP70 genes (Kim, Heo et al. 2011).

In order to see whether a similar mechanism also takes place in other genes, the genome-wide distribution pattern of H3.3 was investigate in this project by ChIP-seq using anti-GFP antibody in a mouse model expressing the tagged version of H3.3 (H3.3B-EGFP) in both HP1 γ WT and HOM MEFs. Rather surprisingly, instead of reducing enrichment of H3.3 proteins, a more pronounced enrichment of H3.3B-EGFP at the regions around the TSS was observed upon HP1 γ knockout. Moreover, the increased enrichment was found in genes that were upregulated, downregulated or unchanged upon HP1 γ knockout. These data indicate that HP1 γ may act to prevent H3.3 loading at the TSS. However, we do not know yet whether

the increased level of H3.3 at the TSS in the upregulated genes might contribute to their increased expression.

Future experiments should focus on revealing genome-wide distribution of HP1 γ and hence identify genes with HP1 γ binding and correlate this with the H3.3 level as well as the transcription changes due to HP1 γ knockout to find out whether removal of HP1 γ might directly affect H3.3 enrichment and gene expression.

4.7 Results in the current study indicate that HP1 γ is not required for heterochromatic silencing of the *FXN* gene in FRDA

The neurodegenerative disease Friedreich's Ataxia (FRDA) is the most commonly inherited ataxia in Caucasians affecting 1 in 30,000 to 50,000 people (Reviewed in - Koeppen 2011, Koeppen 2013). However, no effective disease modifying treatment for FRDA is available to date (Reviewed in - Libri, Yandim et al. 2014).

A number of previous studies provided evidence suggesting that abnormal *FXN* silencing induced by the GAA triplet-repeat expansions in FRDA is at least partly regulated by epigenetic mechanisms (Reviewed in - Schmucker and Puccio 2010, Yandim, Natisvili et al. 2013). In particular, enrichment of the silencing histone marks H3K9me3 and H3K27me3 on the *FXN* gene suggests heterochromatinisation of the *FXN* gene locus. Intriguingly, it has also been shown that silencing of the *FXN* gene in FRDA in various model systems as well as FRDA patients can be reversed by HDAC inhibitor which led to modification of the histone modification signature at the *FXN* gene locus (Chan, Torres et al. 2013, Libri, Yandim et al. 2014, Soragni, Miao et al. 2014).

A previous study from our group showed that GAA-repeat induced silencing in mammals can be modulated *in vivo* by changing the dose of the reader of H3K9me3 HP1 β , implicating HP1 β as a potential disease modifier (Saveliev, Everett et al. 2003). In line with this finding, higher levels of the HP1 isoform HP1 γ at the *FXN* gene locus has been shown in FRDA fibroblast cell lines in a previous study (De Biase, Chutake et al. 2009). This result suggested that HP1 γ may play a role in abnormal silencing of the *FXN* gene in FRDA. Thus, the role of HP1 γ has then been investigated further in the current study.

Data in the current study indicated that shRNA-mediated knockdown of HP1 γ might have led to an increase in *FXN* mRNA level in the two healthy fibroblast cell lines (GM07492 and GM04503) and one of the FRDA lines (GM03665). However, data were not statistically significant due to high variability between experiments. Upregulation of *FXN* mRNA has also been observed in GM03665 when treated with control scramble or empty vectors indicating the potential non-specific effect

of the technique. Increased FXN mRNA level did not lead to increased frataxin protein level in these cells. Dipstick analysis showed no increase in frataxin protein level in all of the healthy and FRDA fibroblasts tested upon HP1 γ knockdown or in cells treated with the control vectors.

The role of HP1 γ was also investigated *in vivo* with a mouse model which carried the *hFXN* transgene with pathologically expanded GAA repeats (Pook, Al-Mahdawi et al. 2001). FXN mRNA level in the cerebella of these mice with heterozygous knockout of HP1 γ were tested. However, no significant change of FXN mRNA was observed. This result was different from the one obtained previously (Figure 3.35A) and might reflect a variation in the GAA-repeat length which has been shown to increase in length across generations (Anjomani Virmouni, Ezzatizadeh et al. 2015).

The result obtained here may be explained by the fact that HP1 γ protein is not completely removed in both of the FRDA models tested. Residual HP1 γ proteins in these models are still functional and may be able to maintain abnormal silencing of the *FXN* gene. To test this hypothesis, alternative FRDA models with complete knockout of HP1 γ are required. However, it was not possible to examine HP1 γ homozygote mice as the gene is essential (Takada, Naruse et al. 2011). Conditional knockout mice which allow homozygous knockout of HP1 γ in FRDA affected tissues would potentially be a better model for this study. Moreover, CRISPR/Cas9 technology which provides efficient knockout may also be employed to target the *CBX3* gene (which encodes HP1 γ) in cell models.

The unchanged frataxin level upon reduction of HP1 γ suggests that removing HP1 γ alone is not sufficient to reverse silencing of *FXN* in FRDA. One might suspect that other isoforms of HP1 may compensate for the HP1 γ deficiency and maintain silencing of the *FXN* gene in FRDA. For example, HP1 β which was implicated in GAA-repeats mediated silencing of a reporter gene (Saveliev, Everett et al. 2003) may potentially play a role in FRDA. Moreover, removal of HP1 γ , a reader of the silencing histone mark H3K9me3 may not be sufficient to reactivate the *FXN* gene in FRDA. Rather, the ‘writers’ of the silencing histone mark H3K9me3 (SUV39H, G9a or SETDB1) or H3K27me3 (components of the polycomb PRC2 complex)

could potentially be targeted to reduce the level of these silencing marks on the *FXN* gene to reverse silencing.

At this stage, the result obtained does not support the hypothesis that HP1 γ is required for maintenance of heterochromatic silencing in FRDA. Further experiments are required to address this hypothesis or to identify alternative factors that mediate silencing of the *FXN* gene in FRDA.

4.8 Future Work

In this thesis, it was shown that HP1 γ has both an activatory and repressive role in the regulation of gene expression and is implicated in a variety of physiological processes. It is also involved in silencing of pericentric Mj-sat transcript which is associated with de-localisation of HP1 β . Of particular interest, males showed a higher dependency on HP1 γ in maintaining their normal gene expression profile compared to females. Our results also suggest that HP1 γ is playing a role in maintaining sexual differences by maintaining the sex-specific expression of the sexually dimorphic genes. Furthermore, preliminary analysis surprisingly revealed increased enrichment level of H3.3 at the TSS in response to HP1 γ knockout. Further experiments are now required to investigate the precise mechanisms whereby HP1 γ might mediate these processes.

Several of the main questions which would be interesting to explore in future studies are discussed in the following sections.

4.8.1 Further investigation into the role of HP1 γ in regulation of sexually dimorphic expression of genes

1) To investigate whether Y-specific genes are contributing to the male-specific repressive role of HP1 γ on a subset of the sexually dimorphic genes and whether X escapees from X-inactivation are having a protective effect in females from HP1 γ mediated repression, CRISPR/Cas9 technology can be employed to knockout these X- or Y-specific genes in MEFs. CRISPR/Cas9 technology allows knockout of genes on the highly heterochromatic Y chromosome which was previously impossible (Wang, Yang et al. 2013). Also, such candidate factors could be overexpressed as transgenes in WT or HP1 γ HOM MEFs. The changes in the 130 sexually dimorphic genes in the cells with the candidate genes knocked out or overexpressed can then be compared to that of the HP1 γ knockout cells. Thus, the Y-specific factors that act in the same pathway with HP1 γ contributing to the repressive effects or the X-specific factors that contribute to a potential ‘protective’ effect against repression by HP1 γ on the 85 genes that are normally expressed at a

lower level in males than female might be identified.

2) To investigate whether the X chromosome is acting as a heterochromatic 'sink' to sequester HP1 γ and lead to the higher expression of the 85 genes in female, a mouse model which contains XIST on an autosome providing a potential autosomal 'sink' (Anton Wutz – collaboration) might be employed. Use of such of an autosomal model would potentially overcome potential problems in testing the 'sink' effect from the inactive X chromosome which would be difficult to be distinguished from the gene escapee effects as mice which possess multiple X chromosomes (XX and XXX) although they would have additional 'sink' due to inactivation of all but one X would also have higher expression of the X chromosome escapees.

3) To investigate whether HP1 γ directly regulates the 130 sexually dimorphic genes, we can take the advantage of the HP1 γ ChIP-seq data when available and test for the binding of HP1 γ on these genes. Moreover, the DNA sequence of these 130 genes can be scanned in an unbiased way using bioinformatics tools (e.g. Bioprospector) to look for common sequence motifs of these genes which may potentially serve as binding sites for HP1 γ or its interacting partner.

4.8.2 Investigation into the context dependent regulation of gene expression by HP1 γ *in vivo*

Given that HP1 β was shown by IF to re-distribute away from pericentromeric heterochromatin to euchromatin (Figure 3.19), it would be interesting to investigate whether HP1 β binds to sites previously occupied by HP1 γ in absence of HP1 γ and whether various HP1 homo- or heterodimers that are composed of different combinations of HP1 proteins may contribute to distinct functions in the genome, we may make use of the transgenic mice carrying HP1 β fused to a biotin tag being developed in the Festenstein lab to reveal genome-wide distribution of HP1 β in presence or absence of HP1 γ .

4.8.3 Investigation into the role of HP1 γ in the regulation of heterochromatin

As HP1 γ deletion was shown in this project to lead to upregulation of the Mj-sat repeats as detected by qRT-PCR, it will be interesting to determine whether the genes nearby pericentric regions were also affected upon HP1 γ knockout. To address this question, genes located in nearby regions of the 42 intergenic Mj-sat sequences interspersed in the mouse genome can be identified using the bioinformatics algorithm developed by Dr. Thomas Carroll (Bioinformatics, MRC CSC, UK). These nearby genes can then be tested for their sensitivity to HP1 γ knockout using the existing RNA-seq data obtained from male and female HP1 γ WT and HOM MEFs.

Appendix

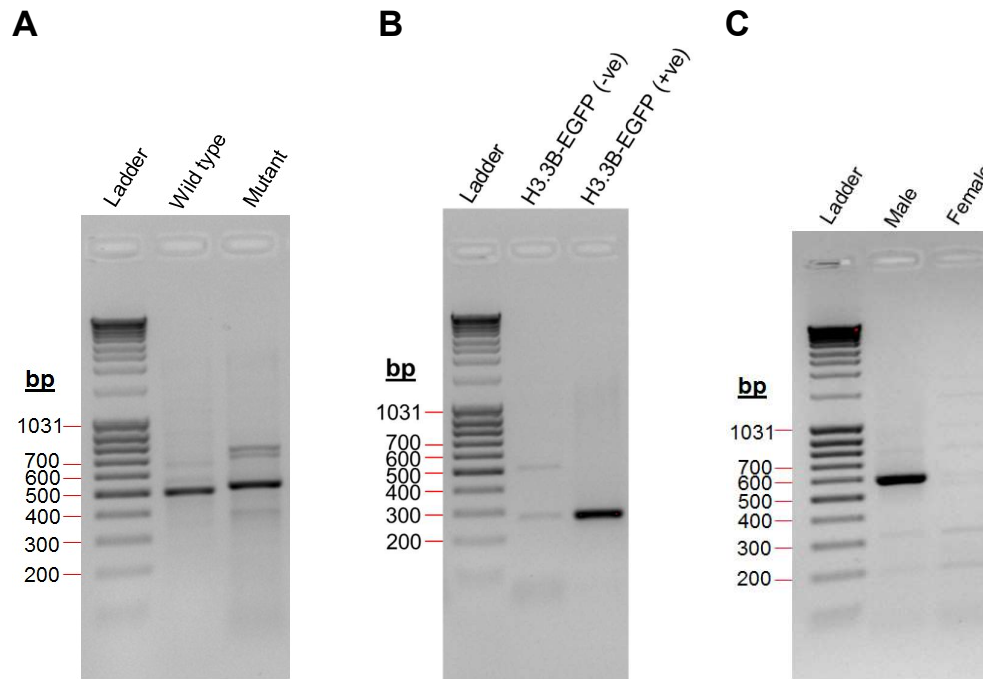


Figure S1 Representative genotyping results. Genotype of transgenic animals or E13.5 embryos were determined by PCR followed by visualisation of PCR products on DNA agarose gel. (A) PCR result for HP1 γ genotyping. PCR for HP1 γ genotyping showed a 501bp band for wild type allele and a 525bp band for mutant allele. (B) PCR result for H3.3B-EGFP genotyping. Animals which express H3.3B-EGFP fusion protein showed a 272bp band while wild type animals show no band on gel. (C) PCR result for Kdm5d genotyping for sex determination of embryos. Male animals showed a 597bp band while female animal showed no band.

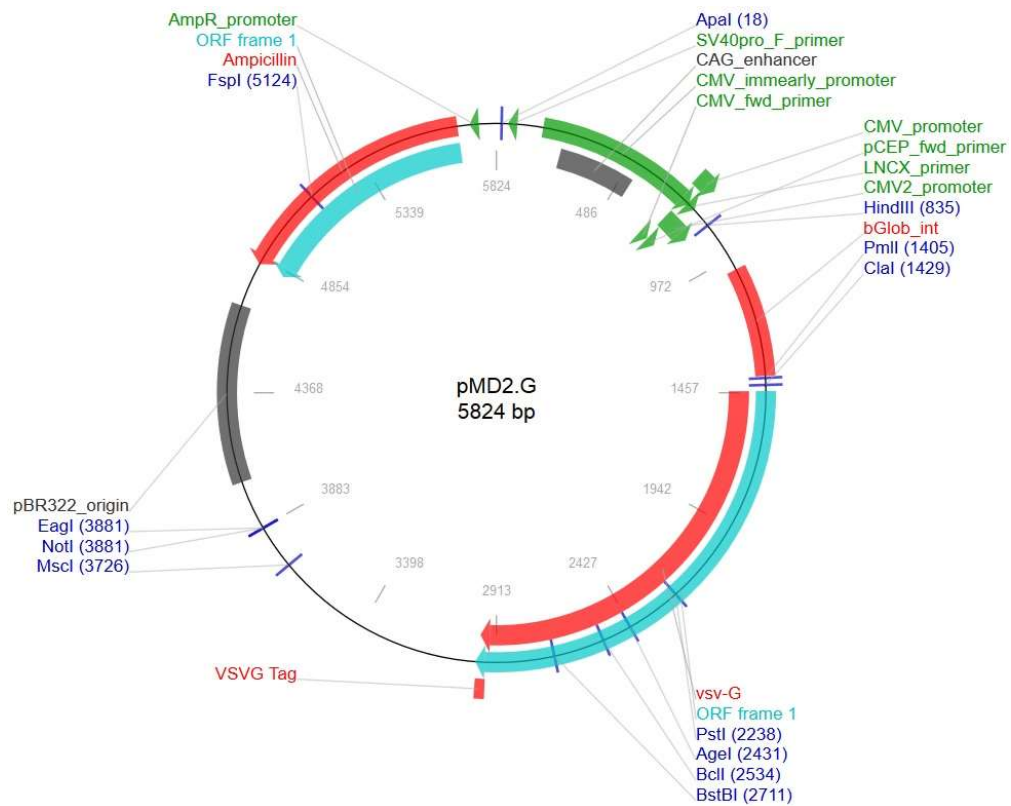


Figure S2 Schematic map of pMD2.G plasmid construct used for shRNA-mediated knockdown experiment. This figure is adapted from Addgene website. pMD2.G was a gift from Didier Trono (Addgene plasmid # 12259, <https://www.addgene.org/12259/>).

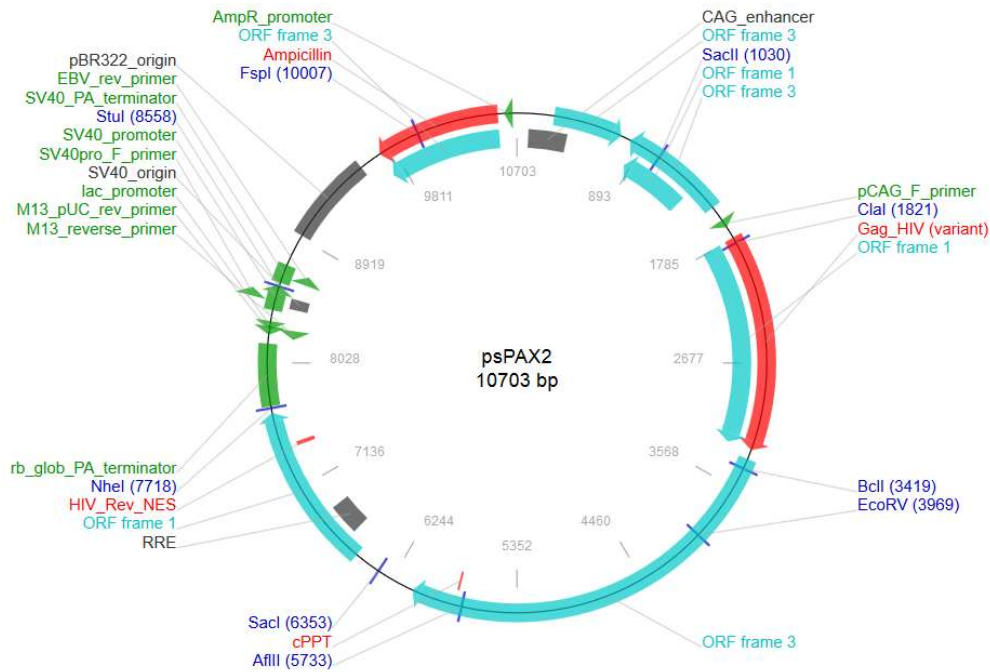


Figure S3 Schematic map of psPAX2 plasmid construct used for shRNA-mediated knockdown experiment. This figure is adapted from Addgene website. psPAX2 was a gift from Didier Trono (Addgene plasmid # 12260, <http://www.addgene.org/12260/>).

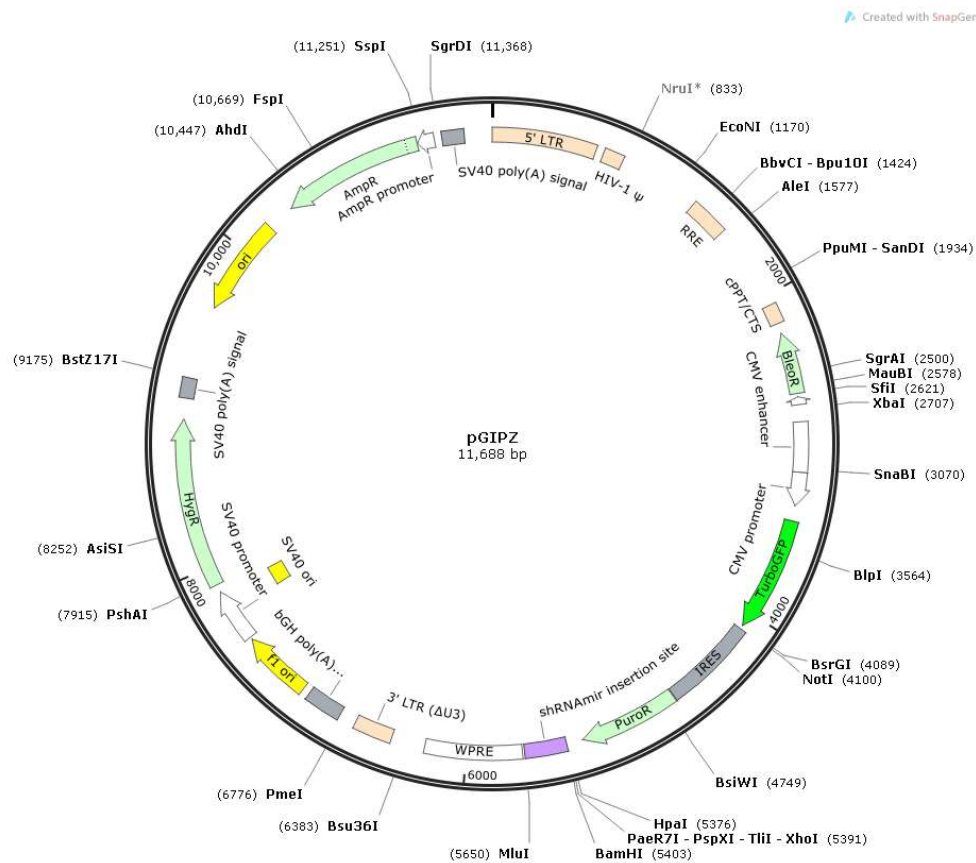


Figure S4 Schematic map of pGIPZ plasmid construct used for shRNA-mediated knockdown experiment. This figure is generated by Snapgene® version 2.3.5 (GSL Biotech).

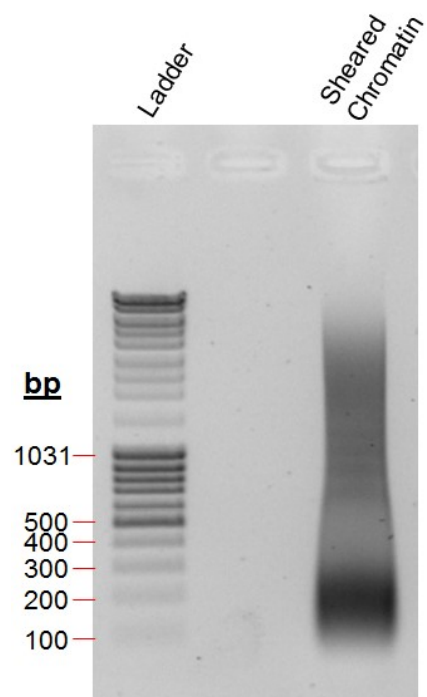


Figure S5 Representative gel for sheared chromatin. Chromatin from MEFs was prepared using Bioruptor® (Diagenode) to obtain sample enriched in chromatin fragments with size of about 150-300bp.

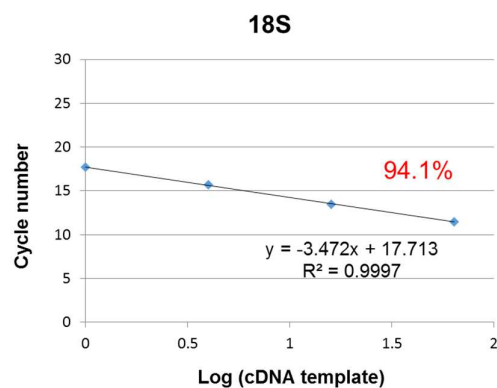
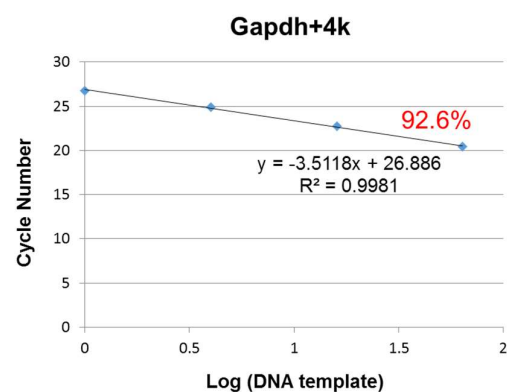
A**B**

Figure S6 Representative standard curve for qRT-PCR primer efficiency test. Standard curves were generated by qRT-PCR on serial diluted DNA samples for calculation of qRT-PCR primer efficiency. Representative standard curve for (A) 18S and (B) Gadph+4k primers were shown here. Efficiency of these primers in percentage were calculated by the formula: Primer efficiency (%) = $(10^{(-1/\text{slope})} - 1) * 100$ and shown in red on the figure.

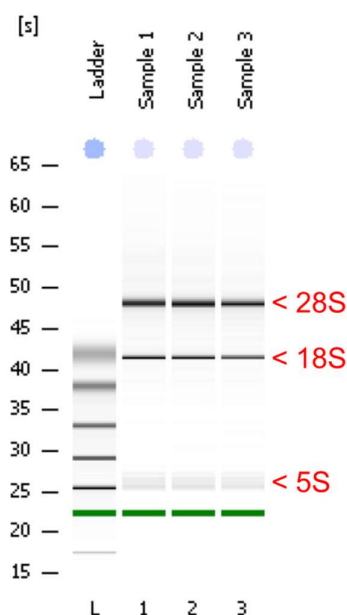
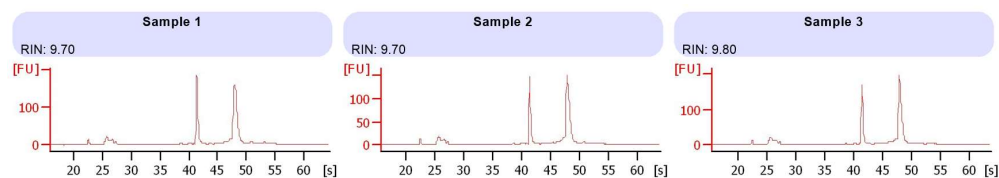
A**B**

Figure S7 Representative result obtained from Bioanalyzer RNA 6000 Nano Assay for checking quality of RNA. Quality of TRIZOL® extracted RNA was checked prior RNA sequencing library preparation. (A) Gel image of RNA showed intact and clear 28S, 18S and 5S rRNA bands which indicate good quality of RNA. (B) Electropherograms of RNA samples and the RNA integrity number (RIN), a classification system of RNA quality developed by Agilent Technologies, were shown for each sample. RIN of 1 refers to the most degraded profile while 10 refers to the most intact. RNA with RIN of ≥ 8.5 were used in this thesis for RNA sequencing library preparation.

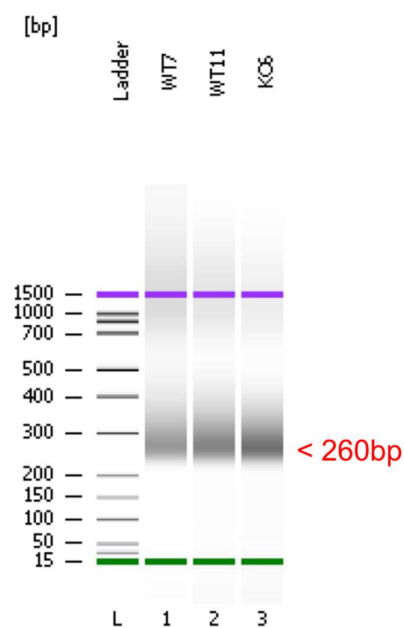
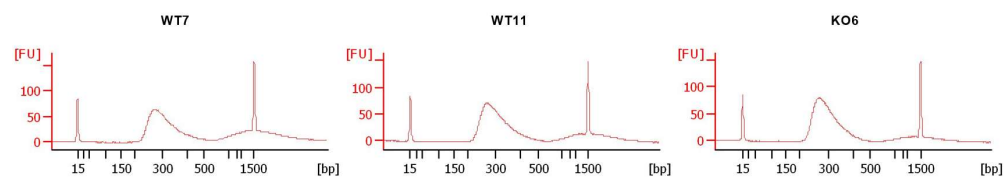
A**B**

Figure S8 Representative result obtained from Bioanalyzer High Sensitivity DNA Analysis for checking size and quality of RNA sequencing library. (A) Gel image and (B) electropherograms of the prepared RNA sequencing library showing DNA fragment a band at approximately at 260bp, indicating good quality of RNA sequencing library as suggested by Illumina® protocol.

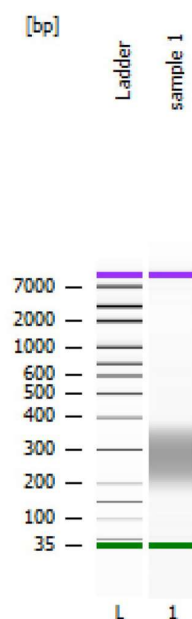
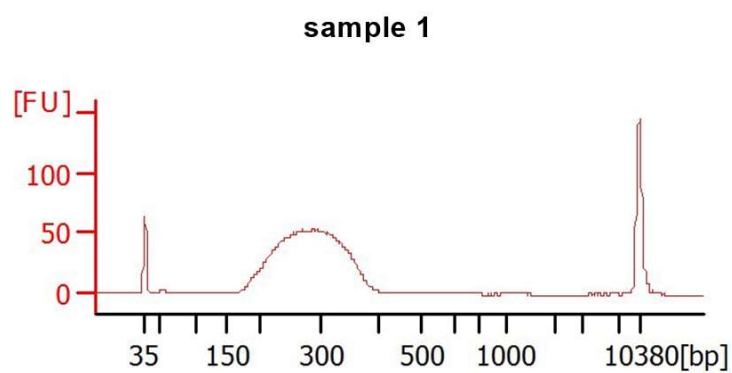
A**B**

Figure S9 Representative result obtained from Bioanalyzer High Sensitivity DNA Analysis for checking size and quality of ChIP sequencing library. (A) Gel image and (B) electropherograms of the prepared ChIP sequencing library showing enrichment of DNA fragment of about 200-400bp which indicate the size of DNA to be sequenced are of about 150-350bp (without ligated adaptors).

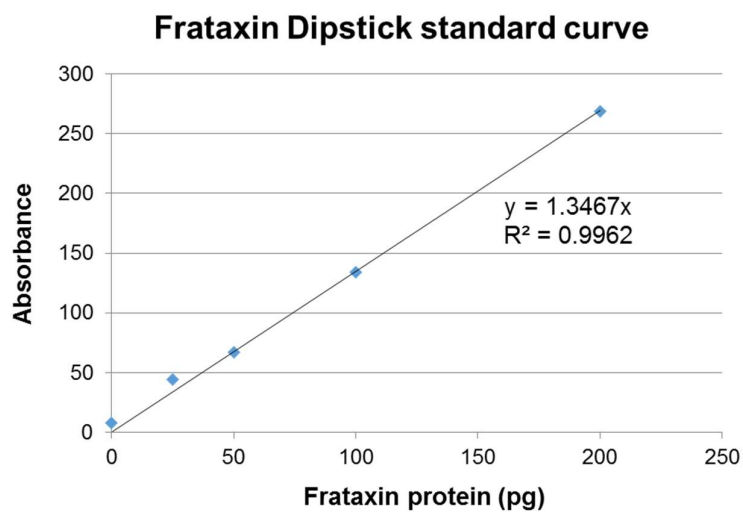


Figure S10 Representative standard curve for frataxin dipstick assay. Standard curves were generated by Quantity Dipstick Assay Kit on serial diluted recombinant frataxin protein using a MS1000 dipstick reader. This standard curve was used to calculate the frataxin protein levels of human fibroblasts protein samples by interpolation.

Relative Expression of HP1 γ in
HP1 γ wild type or heterozygous knockout thymocytes

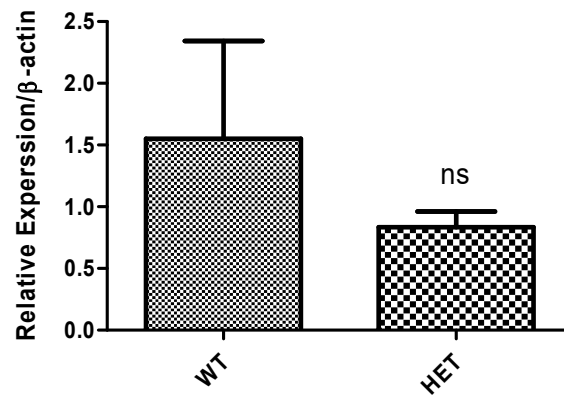


Figure S11 Relative expression of HP1 γ mRNA in HP1 γ WT and heterozygous knockout (HET) thymocytes. In average, HP1 γ mRNA level in HP1 γ HET thymocytes was reduced by half (54% of WT remaining). However, the change is not significant due to variation of data. Student's t-test: ns = not significant (compared to WT after being normalised against β -actin expression). Error bars: SEM, $n \geq 3$.

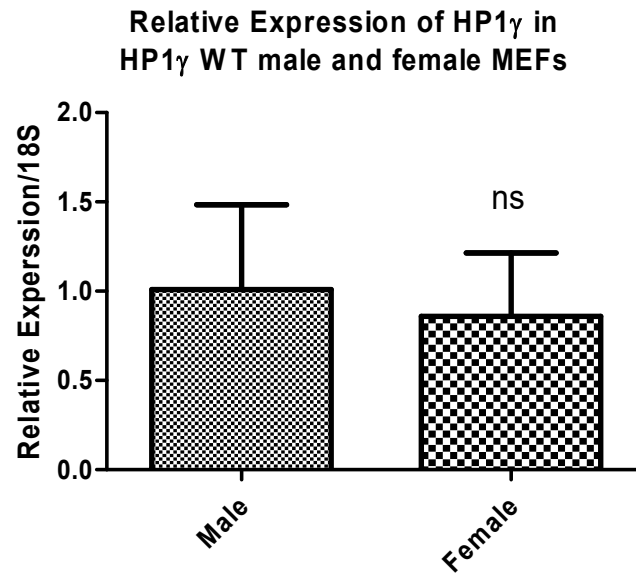


Figure S12 Relative expression of HP1 γ mRNA in HP1 γ WT male and female MEFs. No significant difference in HP1 γ mRNA expression level was observed in male and female WT MEFs. Student's t-test: ns = not significant (compared to male after being normalised against 18S expression). Error bars: SD, $n \geq 10$.

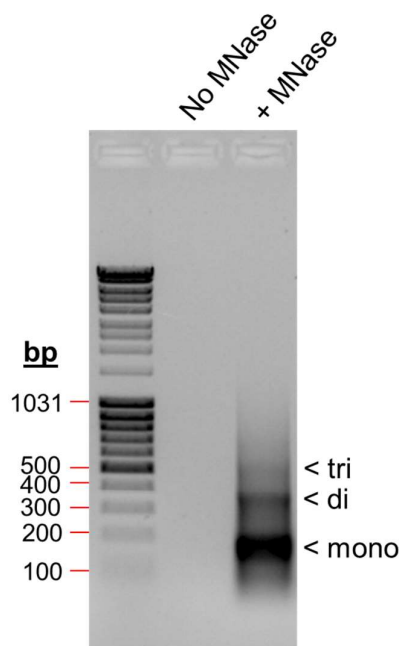
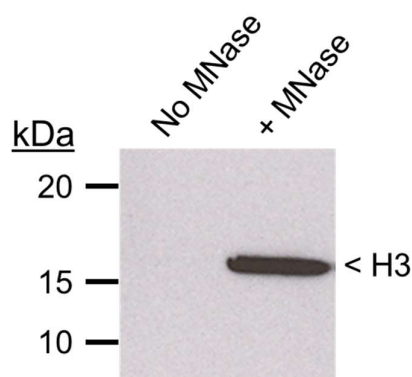
A**B**

Figure S13 Micrococcal nuclease (MNase) and high salt treatment enriched chromatin fraction of histone in MEFs nuclear lysate. (A) Representative gel image of the phenol/chloroform purified DNA obtained from MEFs nuclear lysate with (right lane) or without (left lane) MNase and followed by high salt (340mM NaCl) treatment. Treatment of MNase enriched chromatin fraction in the nuclear lysate as indicated by the recovered DNA of size of mono-, di- and tri-nucleosome. In contrast, no DNA was recovered in nuclear lysate without MNase treatment. (B) Western blot analysis of nuclear lysate with (right lane) or without (left lane) MNase and followed by high salt (340mM NaCl) treatment using anti-H3 antibody. Treatment of MNase enriched histone H3 recovery in the nuclear lysate while there is no detectable histone H3 in nuclear lysate without MNase treatment. This result indicates the enrichment of chromatin fraction with MNase treatment.

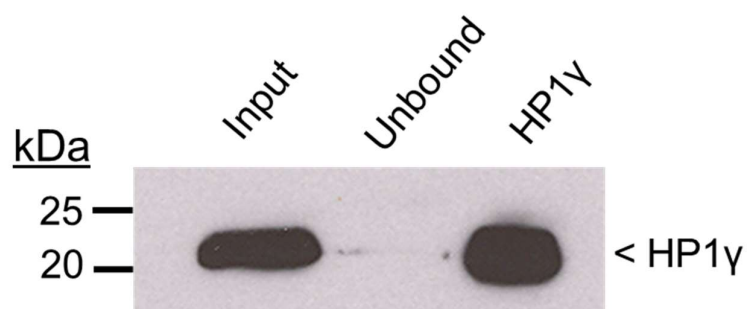
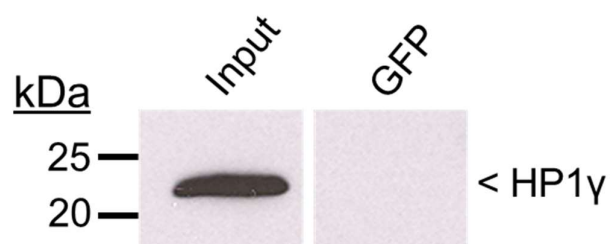
A**B**

Figure S14 Validation of co-immunoprecipitation experiment. (A) HP1 γ protein was enriched in the pulled-down fraction prepared using anti-HP1 γ antibody and diminished in the unbound fraction. This result indicates the high efficiency of pull down. (B) Co-immunoprecipitation experiment in WT MEFs using anti-GFP antibody revealed no HP1 γ in the pulled-down fraction indicates the signal detected between H3.3B-EGFP and HP1 γ in H3.3B-EGFP MEFs was not an artifact.

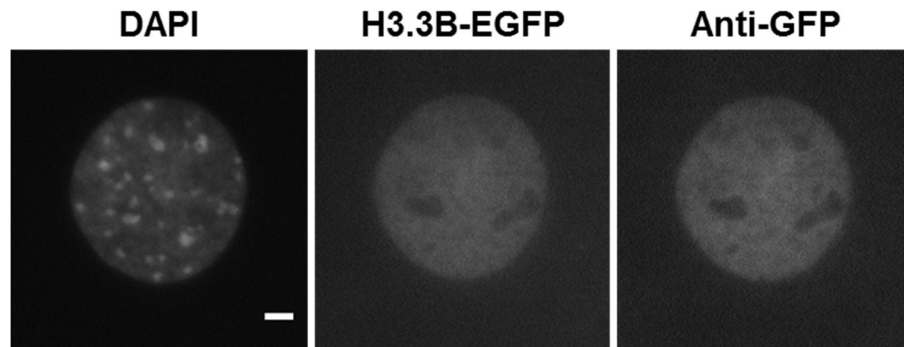


Figure S15 Immunofluorescence analysis of H3.3B-EGFP protein in WT HP1 γ H3.3B-EGFP MEFs. H3.3B-EGFP was found predominately in euchromatin regions and excluded from DAPI-dense regions in most cases. Pattern of endogenous H3.3B-EGFP was very similar to that revealed by anti-GFP antibody staining. Scale bar, 5 μ m. These images were generated by Leica widefield fluorescence microscope and analysed with ImageJ.

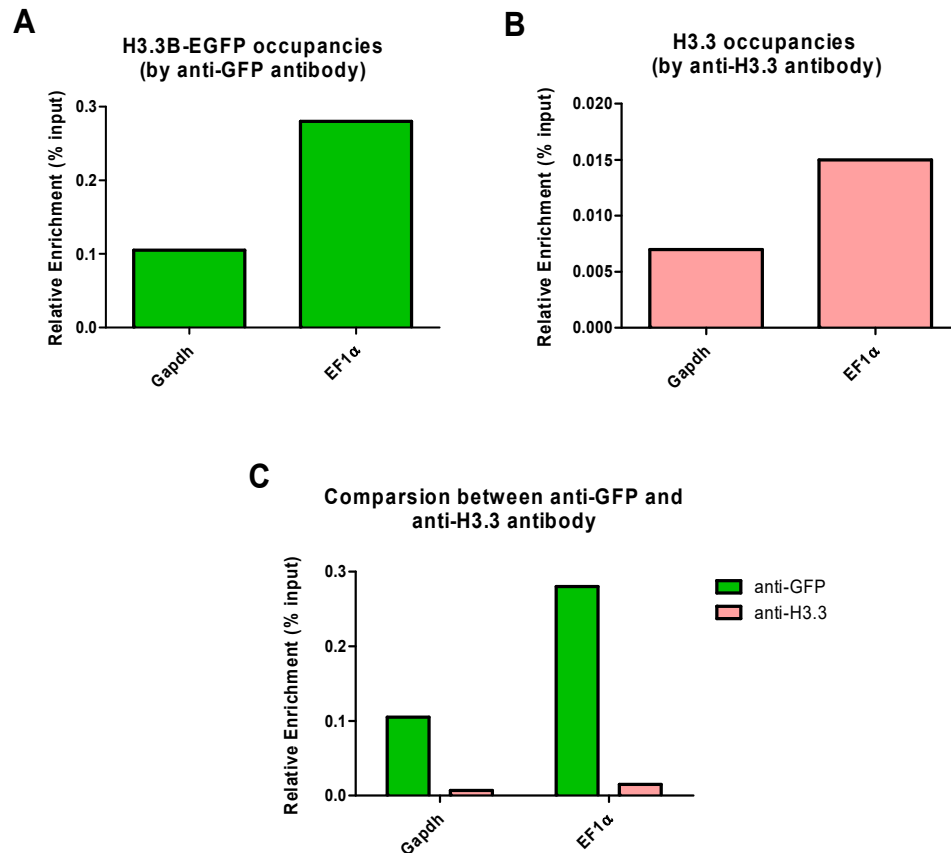


Figure S16 Relative enrichment of H3.3 on various genomic regions in WT HP1 γ H3.3B-EGFP MEFs. (A & B) Enrichment of H3.3 signal on the promoter of the active housekeeping genes *EF1 α* and *Gapdh* revealed by anti-H3.3 antibody (B) are comparable to the H3.3B-EGFP signal revealed anti-GFP antibody (A). (C) Level of H3.3 enrichment (in percentage of total input) obtained by anti-GFP antibody was much higher than that of the anti-H3.3 antibody. n=1.

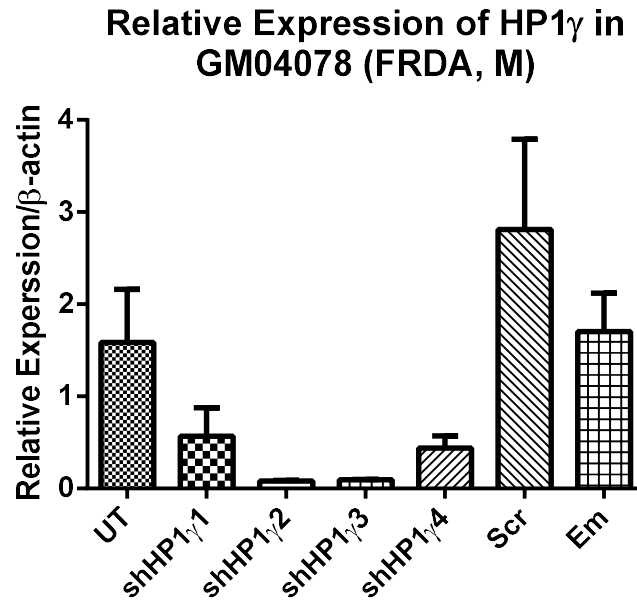


Figure S17 Screening of HP1 γ -targeting shRNA constructs in human fibroblast cell line. Four HP1 γ -targeting shRNA lentiviral constructs (shHP1 γ 1, shHP1 γ 2, shHP1 γ 3 and shHP1 γ 4) were screened using fibroblast cell line GM04078. shHP1 γ 1 and shHP1 γ 2 which reduce HP1 γ to different extent were employed for the full experiment (see result section 3.4.1). Non-targeting control with scrambled shRNA sequence (Scr) or no shRNA insert (Em) were also included. Error bars: SEM, n=2.

Table S1 Biological processes (BP) revealed by gene ontology (GO) analysis associated with upregulated genes upon HP1 γ knockout in male MEFs. The number of 'count' represent the number of dyregulated genes contribute to each term. Adjusted P-value <10⁻⁵.

	GOBPID	Adjusted P-value	Count	Term
1.	GO:0030036	3.85E-05	139	actin cytoskeleton organisation
2.	GO:0030029	2.10E-05	150	actin filament-based process
3.	GO:0002253	1.80E-15	111	activation of immune response
4.	GO:0002218	8.34E-12	55	activation of innate immune response
5.	GO:0002250	1.51E-07	92	adaptive immune response
6.	GO:0002460	4.06E-09	84	adaptive immune response based on somatic recombination of immune receptors built from immunoglobulin superfamily domains
7.	GO:0006066	2.16E-06	95	alcohol metabolic process
8.	GO:0046631	3.77E-05	42	alpha-beta T cell activation
9.	GO:0042886	9.88E-05	75	amide transport
10.	GO:0006820	8.64E-05	129	anion transport
11.	GO:0019882	4.83E-07	47	antigen processing and presentation
12.	GO:0019884	5.54E-07	19	antigen processing and presentation of exogenous antigen
13.	GO:0002478	7.44E-09	18	antigen processing and presentation of exogenous peptide antigen
14.	GO:0019886	2.36E-05	11	antigen processing and presentation of exogenous peptide antigen via MHC class II
15.	GO:0048002	1.30E-06	32	antigen processing and presentation of peptide antigen
16.	GO:0002495	8.37E-07	14	antigen processing and presentation of peptide antigen via MHC class II
17.	GO:0002504	1.59E-05	14	antigen processing and presentation of peptide or polysaccharide antigen via MHC class II
18.	GO:0050851	4.34E-05	37	antigen receptor-mediated signaling pathway
19.	GO:0006915	1.99E-07	424	apoptotic process
20.	GO:0097190	6.01E-05	151	apoptotic signaling pathway
21.	GO:0019439	1.41E-07	291	aromatic compound catabolic process
22.	GO:0006914	1.06E-08	60	autophagy
23.	GO:0042113	3.30E-06	81	B cell activation
24.	GO:0050853	1.57E-06	21	B cell receptor signaling pathway
25.	GO:0007610	4.93E-06	194	behaviour
26.	GO:0022610	5.75E-09	279	biological adhesion

27.	GO:0007596	4.40E-05	52	blood coagulation
28.	GO:0006816	9.33E-05	99	calcium ion transport
29.	GO:0019722	2.21E-06	42	calcium-mediated signaling
30.	GO:1901136	1.01E-13	270	carbohydrate derivative catabolic process
31.	GO:1901135	2.25E-14	445	carbohydrate derivative metabolic process
32.	GO:0005975	1.07E-08	198	carbohydrate metabolic process
33.	GO:0046395	2.91E-05	62	carboxylic acid catabolic process
34.	GO:0019752	4.85E-06	220	carboxylic acid metabolic process
35.	GO:0046942	6.33E-05	77	carboxylic acid transport
36.	GO:0009056	8.10E-23	659	catabolic process
37.	GO:0006812	1.44E-08	272	cation transport
38.	GO:0001775	2.74E-16	254	cell activation
39.	GO:0002263	7.62E-07	71	cell activation involved in immune response
40.	GO:0007155	5.37E-09	277	cell adhesion
41.	GO:0060326	1.85E-05	66	cell chemotaxis
42.	GO:0008219	5.13E-08	456	cell death
43.	GO:0030154	1.39E-05	769	cell differentiation
44.	GO:0016477	4.66E-10	291	cell migration
45.	GO:0000902	3.44E-05	258	cell morphogenesis
46.	GO:0048870	1.32E-09	309	cell motility
47.	GO:0030030	2.66E-08	294	cell projection organisation
48.	GO:0008283	9.08E-07	429	cell proliferation
49.	GO:0016337	4.85E-05	111	cell-cell adhesion
50.	GO:0007267	1.81E-06	208	cell-cell signaling
51.	GO:0043603	3.89E-08	57	cellular amide metabolic process
52.	GO:0044248	1.68E-17	564	cellular catabolic process
53.	GO:0032989	5.96E-05	273	cellular component morphogenesis
54.	GO:0006928	2.59E-10	386	cellular component movement
55.	GO:0016043	1.35E-05	1052	cellular component organisation
56.	GO:0048869	3.37E-06	823	cellular developmental process
57.	GO:0044242	4.03E-08	62	cellular lipid catabolic process
58.	GO:0044255	1.57E-15	257	cellular lipid metabolic process
59.	GO:0051641	2.92E-12	578	cellular localisation
60.	GO:0044237	1.08E-07	2029	cellular metabolic process
61.	GO:0006575	4.25E-05	59	cellular modified amino acid metabolic process
62.	GO:0044270	1.09E-07	289	cellular nitrogen compound catabolic process
63.	GO:0006464	7.89E-05	637	cellular protein modification process
64.	GO:0070887	5.41E-10	469	cellular response to chemical stimulus
65.	GO:0071345	3.30E-06	115	cellular response to cytokine stimulus
66.	GO:0071310	3.54E-06	354	cellular response to organic substance
67.	GO:0071356	1.80E-06	31	cellular response to tumor necrosis

				factor
68.	GO:0006672	6.19E-05	28	ceramide metabolic process
69.	GO:0048878	4.46E-08	231	chemical homeostasis
70.	GO:0006935	3.52E-06	136	chemotaxis
71.	GO:0050817	2.10E-05	54	coagulation
72.	GO:0051187	6.04E-05	11	cofactor catabolic process
73.	GO:0001816	2.94E-13	179	cytokine production
74.	GO:0002367	7.98E-05	25	cytokine production involved in immune response
75.	GO:0050663	6.64E-06	48	cytokine secretion
76.	GO:0016265	7.65E-08	456	death
77.	GO:0006952	7.58E-18	330	defense response
78.	GO:0098542	4.44E-06	113	defense response to other organism
79.	GO:0051607	1.31E-05	59	defense response to virus
80.	GO:0032502	1.47E-05	1149	developmental process
81.	GO:0006897	7.09E-18	167	endocytosis
82.	GO:0007173	1.19E-05	30	epidermal growth factor receptor signaling pathway
83.	GO:0038127	4.66E-06	33	ERBB signaling pathway
84.	GO:0070371	7.89E-05	58	ERK1 and ERK2 cascade
85.	GO:0051234	5.83E-37	1134	establishment of localisation
86.	GO:0051649	3.77E-13	511	establishment of localisation in cell
87.	GO:0045184	1.08E-13	397	establishment of protein localisation
88.	GO:0006887	6.39E-09	92	exocytosis
89.	GO:0097191	4.85E-06	78	extrinsic apoptotic signaling pathway
90.	GO:0006631	8.33E-07	106	fatty acid metabolic process
91.	GO:0048699	2.19E-05	315	generation of neurons
92.	GO:0002467	6.04E-05	11	germinal center formation
93.	GO:0006749	2.19E-06	24	glutathione metabolic process
94.	GO:0046486	1.60E-09	97	glycerolipid metabolic process
95.	GO:0006650	3.58E-08	71	glycerophospholipid metabolic process
96.	GO:1901658	2.94E-10	236	glycosyl compound catabolic process
97.	GO:1901657	5.21E-09	267	glycosyl compound metabolic process
98.	GO:0006184	2.80E-10	178	GTP catabolic process
99.	GO:0046039	3.06E-10	181	GTP metabolic process
100.	GO:1901069	9.08E-11	181	guanosine-containing compound catabolic process
101.	GO:1901068	4.18E-10	183	guanosine-containing compound metabolic process
102.	GO:0007599	1.67E-05	54	hemostasis
103.	GO:0046700	8.48E-08	291	heterocycle catabolic process
104.	GO:0042592	1.09E-08	352	homeostatic process
105.	GO:0007249	8.05E-10	83	I-kappaB kinase/NF-kappaB signaling

106.	GO:0002252	3.83E-16	190	immune effector process
107.	GO:0006955	8.06E-30	326	immune response
108.	GO:0002429	2.23E-06	45	immune response-activating cell surface receptor signaling pathway
109.	GO:0002757	6.31E-15	93	immune response-activating signal transduction
110.	GO:0002768	9.06E-07	50	immune response-regulating cell surface receptor signaling pathway
111.	GO:0002764	1.90E-15	99	immune response-regulating signaling pathway
112.	GO:0002376	3.31E-35	585	immune system process
113.	GO:0006954	1.18E-12	171	inflammatory response
114.	GO:0045087	1.33E-17	164	innate immune response
115.	GO:0002758	5.13E-11	51	innate immune response-activating signal transduction
116.	GO:0048017	7.89E-05	41	inositol lipid-mediated signaling
117.	GO:0032607	6.04E-05	11	interferon-alpha production
118.	GO:0032608	6.93E-06	20	interferon-beta production
119.	GO:0032635	2.19E-06	41	interleukin-6 production
120.	GO:0035556	6.25E-29	630	intracellular signal transduction
121.	GO:0046907	5.54E-07	317	intracellular transport
122.	GO:0050801	3.30E-06	166	ion homeostasis
123.	GO:0034220	1.79E-06	218	ion transmembrane transport
124.	GO:0006811	1.22E-13	397	ion transport
125.	GO:0007254	5.11E-05	56	JNK cascade
126.	GO:0045321	1.19E-14	224	leukocyte activation
127.	GO:0002366	7.62E-07	71	leukocyte activation involved in immune response
128.	GO:0007159	4.02E-05	21	leukocyte cell-cell adhesion
129.	GO:0030595	3.29E-06	54	leukocyte chemotaxis
130.	GO:0043299	2.91E-05	22	leukocyte degranulation
131.	GO:0002521	3.78E-07	144	leukocyte differentiation
132.	GO:0002443	3.40E-08	91	leukocyte mediated immunity
133.	GO:0050900	6.44E-09	85	leukocyte migration
134.	GO:0070661	1.53E-06	90	leukocyte proliferation
135.	GO:0008610	4.28E-06	150	lipid biosynthetic process
136.	GO:0016042	2.86E-12	102	lipid catabolic process
137.	GO:0010876	1.44E-06	72	lipid localisation
138.	GO:0006629	2.47E-21	365	lipid metabolic process
139.	GO:0030258	5.38E-08	64	lipid modification
140.	GO:0006869	6.99E-07	84	lipid transport
141.	GO:0051179	6.81E-39	1364	localisation
142.	GO:0051674	9.59E-06	189	localisation of cell
143.	GO:0040011	1.37E-11	371	locomotion
144.	GO:0007626	8.69E-06	72	locomotory behaviour
145.	GO:0046649	3.07E-10	183	lymphocyte activation
146.	GO:0002449	5.80E-05	65	lymphocyte mediated immunity
147.	GO:0072676	6.70E-05	22	lymphocyte migration
148.	GO:0046651	3.37E-06	86	lymphocyte proliferation

149.	GO:0007040	8.22E-09	23	lysosome organisation
150.	GO:0033036	1.42E-13	546	macromolecule localisation
151.	GO:0042116	9.16E-05	21	macrophage activation
152.	GO:0000165	5.97E-10	187	MAPK cascade
153.	GO:0045576	5.35E-07	27	mast cell activation
154.	GO:0043303	8.41E-05	17	mast cell degranulation
155.	GO:0006643	2.47E-05	51	membrane lipid metabolic process
156.	GO:0061024	2.09E-06	143	membrane organisation
157.	GO:0006509	6.93E-06	20	membrane protein ectodomain proteolysis
158.	GO:0033619	6.80E-06	22	membrane protein proteolysis
159.	GO:0008152	2.70E-10	2387	metabolic process
160.	GO:0030001	5.74E-06	207	metal ion transport
161.	GO:0032787	3.27E-06	132	monocarboxylic acid metabolic process
162.	GO:0032943	2.19E-06	87	mononuclear cell proliferation
163.	GO:0002275	2.95E-07	29	myeloid cell activation involved in immune response
164.	GO:0002274	3.25E-11	63	myeloid leukocyte activation
165.	GO:0002444	3.74E-07	30	myeloid leukocyte mediated immunity
166.	GO:0097529	9.15E-06	46	myeloid leukocyte migration
167.	GO:0043066	1.63E-05	183	negative regulation of apoptotic process
168.	GO:0048519	3.96E-10	933	negative regulation of biological process
169.	GO:0043086	1.26E-05	184	negative regulation of catalytic activity
170.	GO:0050866	6.49E-07	54	negative regulation of cell activation
171.	GO:0010648	2.35E-10	267	negative regulation of cell communication
172.	GO:0060548	4.19E-05	197	negative regulation of cell death
173.	GO:0048523	6.36E-10	844	negative regulation of cellular process
174.	GO:0045744	1.12E-05	29	negative regulation of G-protein coupled receptor protein signaling pathway
175.	GO:0002683	8.56E-10	93	negative regulation of immune system process
176.	GO:0032715	6.51E-05	14	negative regulation of interleukin-6 production
177.	GO:1902532	1.51E-06	105	negative regulation of intracellular signal transduction
178.	GO:0002695	1.22E-06	51	negative regulation of leukocyte activation
179.	GO:0045833	3.03E-05	27	negative regulation of lipid metabolic process
180.	GO:0051250	1.67E-05	43	negative regulation of lymphocyte activation
181.	GO:0044092	1.22E-06	238	negative regulation of molecular function

182.	GO:0032088	3.32E-05	29	negative regulation of NF-kappaB transcription factor activity
183.	GO:0045936	1.67E-05	110	negative regulation of phosphate metabolic process
184.	GO:0010563	1.67E-05	110	negative regulation of phosphorus metabolic process
185.	GO:0042326	2.09E-05	98	negative regulation of phosphorylation
186.	GO:0043069	4.22E-05	184	negative regulation of programmed cell death
187.	GO:0001933	9.74E-05	81	negative regulation of protein phosphorylation
188.	GO:0048585	1.39E-12	313	negative regulation of response to stimulus
189.	GO:0009968	1.54E-10	255	negative regulation of signal transduction
190.	GO:0023057	4.83E-10	265	negative regulation of signaling
191.	GO:0007399	2.04E-06	448	nervous system development
192.	GO:0022008	7.82E-06	336	neurogenesis
193.	GO:0048666	1.81E-05	176	neuron development
194.	GO:0030182	8.26E-05	282	neuron differentiation
195.	GO:0031175	1.07E-06	202	neuron projection development
196.	GO:0007269	7.58E-05	36	neurotransmitter secretion
197.	GO:0006836	9.05E-06	53	neurotransmitter transport
198.	GO:0034655	2.08E-06	272	nucleobase-containing compound catabolic process
199.	GO:0055086	2.42E-09	343	nucleobase-containing small molecule metabolic process
200.	GO:0009164	8.59E-10	232	nucleoside catabolic process
201.	GO:0009116	9.06E-09	262	nucleoside metabolic process
202.	GO:1901292	4.16E-09	245	nucleoside phosphate catabolic process
203.	GO:0006753	1.54E-09	334	nucleoside phosphate metabolic process
204.	GO:0009143	6.85E-09	225	nucleoside triphosphate catabolic process
205.	GO:0009141	1.79E-09	243	nucleoside triphosphate metabolic process
206.	GO:0009166	5.21E-09	242	nucleotide catabolic process
207.	GO:0009117	2.13E-09	331	nucleotide metabolic process
208.	GO:0016054	2.91E-05	62	organic acid catabolic process
209.	GO:0006082	7.89E-06	235	organic acid metabolic process
210.	GO:0015849	8.49E-05	77	organic acid transport
211.	GO:0015711	1.08E-05	103	organic anion transport
212.	GO:1901361	5.38E-08	301	organic cyclic compound catabolic process
213.	GO:1901615	2.85E-08	133	organic hydroxy compound metabolic process
214.	GO:0015850	9.09E-05	53	organic hydroxy compound transport
215.	GO:1901575	1.99E-17	590	organic substance catabolic process

216.	GO:0071704	2.06E-06	2145	organic substance metabolic process
217.	GO:0071702	1.80E-18	593	organic substance transport
218.	GO:1901565	9.08E-14	309	organonitrogen compound catabolic process
219.	GO:1901564	7.34E-15	529	organonitrogen compound metabolic process
220.	GO:0090407	9.19E-06	135	organophosphate biosynthetic process
221.	GO:0046434	3.03E-10	265	organophosphate catabolic process
222.	GO:0019637	1.37E-17	449	organophosphate metabolic process
223.	GO:0043436	6.29E-06	232	oxoacid metabolic process
224.	GO:0002221	4.03E-11	50	pattern recognition receptor signaling pathway
225.	GO:0006518	7.43E-09	44	peptide metabolic process
226.	GO:0006909	3.07E-10	56	phagocytosis
227.	GO:0090382	6.85E-05	10	phagosome maturation
228.	GO:0006796	5.56E-27	919	phosphate-containing compound metabolic process
229.	GO:0046488	4.96E-07	49	phosphatidylinositol metabolic process
230.	GO:0006644	2.18E-08	95	phospholipid metabolic process
231.	GO:0006793	5.33E-26	926	phosphorus metabolic process
232.	GO:0016310	9.08E-14	517	phosphorylation
233.	GO:0030168	1.80E-06	31	platelet activation
234.	GO:0002821	5.56E-07	36	positive regulation of adaptive immune response
235.	GO:0002824	2.16E-06	33	positive regulation of adaptive immune response based on somatic recombination of immune receptors built from immunoglobulin superfamily domains
236.	GO:0050871	8.33E-07	32	positive regulation of B cell activation
237.	GO:0030890	1.53E-05	21	positive regulation of B cell proliferation
238.	GO:0048518	5.79E-18	1083	positive regulation of biological process
239.	GO:0043085	8.38E-09	257	positive regulation of catalytic activity
240.	GO:0050867	9.28E-08	85	positive regulation of cell activation
241.	GO:0010647	2.16E-09	311	positive regulation of cell communication
242.	GO:0008284	5.35E-06	212	positive regulation of cell proliferation
243.	GO:0048522	1.09E-12	938	positive regulation of cellular process
244.	GO:0032270	4.18E-05	223	positive regulation of cellular protein metabolic process
245.	GO:0032722	6.19E-05	18	positive regulation of chemokine

				production
246.	GO:0001819	1.23E-07	84	positive regulation of cytokine production
247.	GO:0050715	4.59E-05	30	positive regulation of cytokine secretion
248.	GO:0031349	7.18E-09	81	positive regulation of defense response
249.	GO:0051094	1.38E-05	243	positive regulation of developmental process
250.	GO:0043547	3.73E-05	64	positive regulation of GTPase activity
251.	GO:0043123	3.75E-06	49	positive regulation of I-kappaB kinase/NF-kappaB signaling
252.	GO:0002699	7.37E-06	52	positive regulation of immune effector process
253.	GO:0050778	2.20E-18	148	positive regulation of immune response
254.	GO:0002684	4.18E-21	216	positive regulation of immune system process
255.	GO:0045089	5.82E-09	61	positive regulation of innate immune response
256.	GO:0045359	4.22E-05	7	positive regulation of interferon-beta biosynthetic process
257.	GO:1902533	4.02E-10	209	positive regulation of intracellular signal transduction
258.	GO:0033674	8.45E-05	113	positive regulation of kinase activity
259.	GO:0002696	3.88E-07	80	positive regulation of leukocyte activation
260.	GO:0070665	2.58E-05	45	positive regulation of leukocyte proliferation
261.	GO:0045834	3.47E-05	44	positive regulation of lipid metabolic process
262.	GO:0051251	6.91E-07	73	positive regulation of lymphocyte activation
263.	GO:0050671	7.58E-05	42	positive regulation of lymphocyte proliferation
264.	GO:0044093	8.49E-10	322	positive regulation of molecular function
265.	GO:0032946	4.49E-05	43	positive regulation of mononuclear cell proliferation
266.	GO:0051240	9.68E-07	176	positive regulation of multicellular organismal process
267.	GO:0051092	1.33E-05	42	positive regulation of NF-kappaB transcription factor activity
268.	GO:0045937	1.06E-05	222	positive regulation of phosphate metabolic process
269.	GO:0010562	1.06E-05	222	positive regulation of phosphorus metabolic process
270.	GO:0042327	9.47E-06	200	positive regulation of phosphorylation
271.	GO:0051247	1.98E-06	255	positive regulation of protein metabolic process
272.	GO:0031401	4.22E-05	198	positive regulation of protein

				modification process
273.	GO:0001934	3.25E-05	168	positive regulation of protein phosphorylation
274.	GO:0048584	4.24E-14	409	positive regulation of response to stimulus
275.	GO:0032321	6.28E-05	31	positive regulation of Rho GTPase activity
276.	GO:0009967	5.63E-09	291	positive regulation of signal transduction
277.	GO:0023056	6.67E-09	307	positive regulation of signaling
278.	GO:0051347	7.89E-05	117	positive regulation of transferase activity
279.	GO:0051050	2.79E-08	200	positive regulation of transport
280.	GO:0044238	3.56E-06	2052	primary metabolic process
281.	GO:0012501	6.67E-08	435	programmed cell death
282.	GO:0008104	4.67E-12	483	protein localisation
283.	GO:0036211	7.89E-05	637	protein modification process
284.	GO:0006468	8.91E-09	377	protein phosphorylation
285.	GO:0009306	2.91E-06	74	protein secretion
286.	GO:0015031	6.26E-14	379	protein transport
287.	GO:0006152	1.61E-09	229	purine nucleoside catabolic process
288.	GO:0042278	1.30E-09	254	purine nucleoside metabolic process
289.	GO:0009146	5.82E-09	224	purine nucleoside triphosphate catabolic process
290.	GO:0009144	1.88E-09	239	purine nucleoside triphosphate metabolic process
291.	GO:0006195	2.48E-08	234	purine nucleotide catabolic process
292.	GO:0006163	1.90E-08	290	purine nucleotide metabolic process
293.	GO:0046130	1.61E-09	229	purine ribonucleoside catabolic process
294.	GO:0046128	1.24E-09	253	purine ribonucleoside metabolic process
295.	GO:0009207	9.46E-09	222	purine ribonucleoside triphosphate catabolic process
296.	GO:0009205	2.18E-09	237	purine ribonucleoside triphosphate metabolic process
297.	GO:0009154	3.05E-09	231	purine ribonucleotide catabolic process
298.	GO:0009150	4.46E-09	284	purine ribonucleotide metabolic process
299.	GO:0072523	9.55E-09	238	purine-containing compound catabolic process
300.	GO:0072521	2.57E-08	300	purine-containing compound metabolic process
301.	GO:0070269	1.17E-05	8	pyroptosis
302.	GO:0007265	4.60E-07	91	Ras protein signal transduction
303.	GO:0072593	1.86E-06	56	reactive oxygen species metabolic process
304.	GO:0006898	7.43E-06	57	receptor-mediated endocytosis

305.	GO:0045055	1.85E-06	28	regulated secretory pathway
306.	GO:0002819	7.28E-09	58	regulation of adaptive immune response
307.	GO:0002822	9.66E-08	52	regulation of adaptive immune response based on somatic recombination of immune receptors built from immunoglobulin superfamily domains
308.	GO:0042981	1.35E-05	323	regulation of apoptotic process
309.	GO:0050864	7.90E-08	47	regulation of B cell activation
310.	GO:0030888	4.99E-06	29	regulation of B cell proliferation
311.	GO:0050855	7.19E-05	9	regulation of B cell receptor signaling pathway
312.	GO:0065008	5.79E-18	752	regulation of biological quality
313.	GO:0050878	8.84E-06	87	regulation of body fluid levels
314.	GO:0009894	8.23E-12	244	regulation of catabolic process
315.	GO:0050790	2.72E-16	501	regulation of catalytic activity
316.	GO:0050865	2.76E-12	145	regulation of cell activation
317.	GO:0030155	4.74E-06	100	regulation of cell adhesion
318.	GO:0010646	7.67E-29	747	regulation of cell communication
319.	GO:0010941	2.57E-05	340	regulation of cell death
320.	GO:0045595	7.25E-06	354	regulation of cell differentiation
321.	GO:0030334	1.98E-07	165	regulation of cell migration
322.	GO:2000145	4.32E-07	169	regulation of cell motility
323.	GO:0031344	1.53E-05	120	regulation of cell projection organisation
324.	GO:0042127	5.24E-07	353	regulation of cell proliferation
325.	GO:0031329	2.54E-12	225	regulation of cellular catabolic process
326.	GO:0051270	5.54E-07	185	regulation of cellular component movement
327.	GO:0051128	2.61E-06	420	regulation of cellular component organisation
328.	GO:0060341	3.90E-09	257	regulation of cellular localisation
329.	GO:0031323	1.65E-05	1134	regulation of cellular metabolic process
330.	GO:0001817	1.49E-10	153	regulation of cytokine production
331.	GO:0002718	4.44E-05	22	regulation of cytokine production involved in immune response
332.	GO:0050707	3.91E-05	41	regulation of cytokine secretion
333.	GO:0031347	9.44E-14	148	regulation of defense response
334.	GO:0050793	7.65E-08	492	regulation of developmental process
335.	GO:0030100	4.34E-07	61	regulation of endocytosis
336.	GO:0070372	5.84E-05	54	regulation of ERK1 and ERK2 cascade
337.	GO:0070201	2.36E-05	124	regulation of establishment of protein localisation
338.	GO:0033124	2.29E-11	137	regulation of GTP catabolic process
339.	GO:0043087	3.08E-11	136	regulation of GTPase activity

340.	GO:0032844	6.93E-06	105	regulation of homeostatic process
341.	GO:0051336	4.18E-10	288	regulation of hydrolase activity
342.	GO:0043122	2.64E-08	72	regulation of I-kappaB kinase/NF-kappaB signaling
343.	GO:0002697	9.38E-09	93	regulation of immune effector process
344.	GO:0050776	1.63E-21	195	regulation of immune response
345.	GO:0002682	4.69E-25	325	regulation of immune system process
346.	GO:0050727	5.83E-07	80	regulation of inflammatory response
347.	GO:0045088	1.50E-09	73	regulation of innate immune response
348.	GO:0032647	2.36E-05	11	regulation of interferon-alpha production
349.	GO:0032648	9.19E-06	19	regulation of interferon-beta production
350.	GO:0032675	3.01E-06	40	regulation of interleukin-6 production
351.	GO:1902531	1.05E-23	439	regulation of intracellular signal transduction
352.	GO:0043269	9.70E-05	151	regulation of ion transport
353.	GO:0043549	3.38E-07	182	regulation of kinase activity
354.	GO:0002694	5.75E-11	133	regulation of leukocyte activation
355.	GO:0043300	4.98E-05	15	regulation of leukocyte degranulation
356.	GO:1902105	5.81E-06	78	regulation of leukocyte differentiation
357.	GO:0002703	3.29E-06	54	regulation of leukocyte mediated immunity
358.	GO:0002685	4.34E-05	44	regulation of leukocyte migration
359.	GO:0070663	1.43E-07	70	regulation of leukocyte proliferation
360.	GO:0050994	2.12E-05	23	regulation of lipid catabolic process
361.	GO:0019216	4.25E-08	91	regulation of lipid metabolic process
362.	GO:0032879	8.51E-17	543	regulation of localisation
363.	GO:0040012	1.56E-07	185	regulation of locomotion
364.	GO:0051249	6.85E-09	112	regulation of lymphocyte activation
365.	GO:0050670	7.62E-07	66	regulation of lymphocyte proliferation
366.	GO:0043405	4.03E-05	75	regulation of MAP kinase activity
367.	GO:0043408	1.44E-08	164	regulation of MAPK cascade
368.	GO:0033003	5.00E-05	17	regulation of mast cell activation
369.	GO:0042391	4.18E-05	105	regulation of membrane potential
370.	GO:0019222	1.66E-05	1290	regulation of metabolic process
371.	GO:0065009	2.19E-18	620	regulation of molecular function
372.	GO:0032944	4.59E-07	67	regulation of mononuclear cell proliferation
373.	GO:2000026	1.99E-07	395	regulation of multicellular organismal development
374.	GO:0051239	5.34E-14	620	regulation of multicellular

				organismal process
375.	GO:0043900	6.30E-06	71	regulation of multi-organism process
376.	GO:0002886	6.39E-06	18	regulation of myeloid leukocyte mediated immunity
377.	GO:0051960	5.54E-06	184	regulation of nervous system development
378.	GO:0050767	3.30E-06	169	regulation of neurogenesis
379.	GO:0045664	4.24E-05	136	regulation of neuron differentiation
380.	GO:0010975	1.93E-05	99	regulation of neuron projection development
381.	GO:0001505	6.27E-07	56	regulation of neurotransmitter levels
382.	GO:0009118	7.09E-10	143	regulation of nucleoside metabolic process
383.	GO:0030811	2.59E-09	140	regulation of nucleotide catabolic process
384.	GO:0006140	8.04E-09	172	regulation of nucleotide metabolic process
385.	GO:0050764	4.18E-05	26	regulation of phagocytosis
386.	GO:0019220	1.76E-15	479	regulation of phosphate metabolic process
387.	GO:0051174	1.34E-15	481	regulation of phosphorus metabolic process
388.	GO:0042325	4.76E-09	316	regulation of phosphorylation
389.	GO:0010543	4.85E-06	16	regulation of platelet activation
390.	GO:0080090	4.84E-05	1100	regulation of primary metabolic process
391.	GO:0002700	8.15E-05	33	regulation of production of molecular mediator of immune response
392.	GO:0043067	1.06E-05	328	regulation of programmed cell death
393.	GO:0045859	1.95E-06	167	regulation of protein kinase activity
394.	GO:0032880	1.32E-05	146	regulation of protein localisation
395.	GO:0051246	3.72E-05	409	regulation of protein metabolic process
396.	GO:0031399	1.66E-05	290	regulation of protein modification process
397.	GO:0001932	5.54E-07	250	regulation of protein phosphorylation
398.	GO:0051223	4.22E-05	113	regulation of protein transport
399.	GO:0033121	2.59E-09	140	regulation of purine nucleotide catabolic process
400.	GO:1900542	1.06E-08	170	regulation of purine nucleotide metabolic process
401.	GO:0032318	7.89E-08	92	regulation of Ras GTPase activity
402.	GO:0046578	1.55E-10	132	regulation of Ras protein signal transduction
403.	GO:0032101	1.37E-11	175	regulation of response to external stimulus
404.	GO:0048583	7.64E-32	836	regulation of response to stimulus

405.	GO:0080134	1.01E-13	257	regulation of response to stress
406.	GO:0035023	1.35E-05	65	regulation of Rho protein signal transduction
407.	GO:0051046	9.88E-08	170	regulation of secretion
408.	GO:0009966	1.28E-25	661	regulation of signal transduction
409.	GO:0023051	6.00E-29	747	regulation of signaling
410.	GO:0051056	4.18E-10	136	regulation of small GTPase mediated signal transduction
411.	GO:0050804	5.50E-05	81	regulation of synaptic transmission
412.	GO:0050863	3.52E-06	77	regulation of T cell activation
413.	GO:0034121	5.37E-05	20	regulation of toll-like receptor signaling pathway
414.	GO:0051338	2.01E-07	190	regulation of transferase activity
415.	GO:0051049	1.33E-13	405	regulation of transport
416.	GO:0002828	2.63E-05	15	regulation of type 2 immune response
417.	GO:0032479	9.06E-07	23	regulation of type I interferon production
418.	GO:0060627	1.21E-06	96	regulation of vesicle-mediated transport
419.	GO:0009607	3.76E-08	198	response to biotic stimulus
420.	GO:0034097	2.21E-07	143	response to cytokine
421.	GO:0042493	7.17E-05	69	response to drug
422.	GO:0043207	8.10E-09	193	response to external biotic stimulus
423.	GO:0009605	2.46E-19	480	response to external stimulus
424.	GO:0009725	9.86E-05	144	response to hormone
425.	GO:0002237	2.01E-05	80	response to molecule of bacterial origin
426.	GO:0010033	7.89E-08	508	response to organic substance
427.	GO:0051707	9.32E-09	191	response to other organism
428.	GO:0006979	2.16E-06	90	response to oxidative stress
429.	GO:1901700	2.68E-06	272	response to oxygen-containing compound
430.	GO:0000302	2.48E-05	46	response to reactive oxygen species
431.	GO:0006950	1.24E-18	736	response to stress
432.	GO:0009266	1.35E-05	41	response to temperature stimulus
433.	GO:0034612	3.71E-06	36	response to tumor necrosis factor
434.	GO:0009615	5.49E-06	70	response to virus
435.	GO:0009611	7.86E-16	256	response to wounding
436.	GO:0042454	1.08E-09	231	ribonucleoside catabolic process
437.	GO:0009119	7.35E-10	258	ribonucleoside metabolic process
438.	GO:0009203	1.07E-08	222	ribonucleoside triphosphate catabolic process
439.	GO:0009199	3.21E-09	237	ribonucleoside triphosphate metabolic process
440.	GO:0009261	3.05E-09	231	ribonucleotide catabolic process
441.	GO:0009259	4.76E-09	286	ribonucleotide metabolic process
442.	GO:0019693	3.72E-09	288	ribose phosphate metabolic process

443.	GO:0019932	1.59E-05	59	second-messenger-mediated signaling
444.	GO:0046903	5.04E-13	268	secretion
445.	GO:0032940	4.98E-13	241	secretion by cell
446.	GO:0019233	2.23E-06	46	sensory perception of pain
447.	GO:0023061	1.07E-05	107	signal release
448.	GO:0023014	2.94E-10	195	signal transduction by phosphorylation
449.	GO:0044711	1.86E-05	312	single-organism biosynthetic process
450.	GO:0044723	2.81E-07	171	single-organism carbohydrate metabolic process
451.	GO:0044712	7.54E-28	473	single-organism catabolic process
452.	GO:0044763	3.08E-07	2752	single-organism cellular process
453.	GO:0044767	1.67E-05	1144	single-organism developmental process
454.	GO:1902582	4.44E-05	249	single-organism intracellular transport
455.	GO:0044710	9.17E-27	1279	single-organism metabolic process
456.	GO:0044699	3.79E-09	3080	single-organism process
457.	GO:0044765	9.96E-32	890	single-organism transport
458.	GO:0007264	5.40E-08	151	small GTPase mediated signal transduction
459.	GO:0044282	2.02E-06	80	small molecule catabolic process
460.	GO:0044281	1.39E-17	650	small molecule metabolic process
461.	GO:0006665	2.36E-05	40	sphingolipid metabolic process
462.	GO:0051403	7.89E-05	63	stress-activated MAPK cascade
463.	GO:0031098	5.80E-05	65	stress-activated protein kinase signaling cascade
464.	GO:0006801	1.35E-05	23	superoxide metabolic process
465.	GO:0007268	5.52E-07	164	synaptic transmission
466.	GO:0048731	5.72E-07	859	system development
467.	GO:0042110	2.51E-07	127	T cell activation
468.	GO:0030217	7.72E-05	68	T cell differentiation
469.	GO:0042098	4.48E-05	55	T cell proliferation
470.	GO:0042330	3.99E-06	136	taxis
471.	GO:0038032	6.93E-06	20	termination of G-protein coupled receptor signaling pathway
472.	GO:0023021	4.83E-07	23	termination of signal transduction
473.	GO:0034162	4.22E-05	7	toll-like receptor 9 signaling pathway
474.	GO:0002224	1.44E-08	37	toll-like receptor signaling pathway
475.	GO:0007169	3.55E-05	125	transmembrane receptor protein tyrosine kinase signaling pathway
476.	GO:0055085	1.34E-09	294	transmembrane transport
477.	GO:0006810	1.80E-37	1117	transport
478.	GO:0042092	2.91E-05	17	type 2 immune response
479.	GO:0032606	1.68E-06	25	type I interferon production
480.	GO:0033559	4.59E-05	30	unsaturated fatty acid metabolic process

481.	GO:0007034	9.88E-05	22	vacuolar transport
482.	GO:0007033	4.18E-10	40	vacuole organisation
483.	GO:0051648	8.47E-05	44	vesicle localisation
484.	GO:0016050	2.58E-05	45	vesicle organisation
485.	GO:0016192	1.27E-18	312	vesicle-mediated transport
486.	GO:0042060	4.39E-05	86	wound healing

Table S2 Biological processes (BP) revealed by gene ontology (GO) analysis associated with downregulated genes upon HP1 γ knockout in male MEFs. The number of 'count' represent the number of dyregulated genes contribute to each term. Adjusted P-value $<10^{-5}$.

	GOBPID	Adjusted P-value	Count	Term
1.	GO:0007568	8.79E-06	44	aging
2.	GO:0000380	1.95E-06	20	alternative mRNA splicing, via spliceosome
3.	GO:0043038	2.76E-10	29	amino acid activation
4.	GO:0031145	2.56E-05	10	anaphase-promoting complex-dependent proteasomal ubiquitin-dependent protein catabolic process
5.	GO:0048856	3.00E-14	969	anatomical structure development
6.	GO:0048646	1.27E-07	249	anatomical structure formation involved in morphogenesis
7.	GO:0009653	2.63E-15	569	anatomical structure morphogenesis
8.	GO:0006915	5.76E-10	399	apoptotic process
9.	GO:0048736	1.21E-05	56	appendage development
10.	GO:0035107	3.51E-06	54	appendage morphogenesis
11.	GO:0019438	1.78E-47	961	aromatic compound biosynthetic process
12.	GO:0019439	1.29E-11	284	aromatic compound catabolic process
13.	GO:0006200	1.20E-07	69	ATP catabolic process
14.	GO:0046034	1.85E-07	78	ATP metabolic process
15.	GO:0043044	3.93E-06	14	ATP-dependent chromatin remodeling
16.	GO:0008608	5.45E-07	14	attachment of spindle microtubules to kinetochore
17.	GO:0009058	2.68E-91	1447	biosynthetic process
18.	GO:0001568	4.64E-06	125	blood vessel development
19.	GO:0048514	2.78E-05	104	blood vessel morphogenesis
20.	GO:0060348	1.25E-05	47	bone development
21.	GO:0060349	6.53E-05	32	bone morphogenesis
22.	GO:0060070	4.46E-05	65	canonical Wnt signaling pathway
23.	GO:1901135	3.89E-06	354	carbohydrate derivative metabolic

				process
24.	GO:0003205	1.54E-05	49	cardiac chamber development
25.	GO:0003206	2.21E-05	44	cardiac chamber morphogenesis
26.	GO:0048738	3.58E-05	59	cardiac muscle tissue development
27.	GO:0072358	2.25E-10	221	cardiovascular system development
28.	GO:0009056	3.78E-12	543	catabolic process
29.	GO:0007569	2.28E-06	31	cell aging
30.	GO:0007049	1.77E-80	539	cell cycle
31.	GO:0007050	8.22E-05	42	cell cycle arrest
32.	GO:0000075	1.59E-15	73	cell cycle checkpoint
33.	GO:0044839	3.51E-06	29	cell cycle G2/M phase transition
34.	GO:0022403	6.36E-05	14	cell cycle phase
35.	GO:0044770	2.04E-22	123	cell cycle phase transition
36.	GO:0022402	2.27E-67	401	cell cycle process
37.	GO:0008219	2.88E-10	426	cell death
38.	GO:0048468	3.22E-06	408	cell development
39.	GO:0030154	6.12E-05	682	cell differentiation
40.	GO:0051301	1.60E-48	306	cell division
41.	GO:0000902	1.86E-05	235	cell morphogenesis
42.	GO:0008283	8.73E-09	401	cell proliferation
43.	GO:0006520	1.91E-05	98	cellular amino acid metabolic process
44.	GO:0006725	4.46E-135	1658	cellular aromatic compound metabolic process
45.	GO:0044249	5.13E-93	1408	cellular biosynthetic process
46.	GO:0044248	1.53E-12	485	cellular catabolic process
47.	GO:0022607	7.55E-26	490	cellular component assembly
48.	GO:0044085	5.54E-45	589	cellular component biogenesis
49.	GO:0022411	8.22E-06	56	cellular component disassembly
50.	GO:0032989	1.02E-06	259	cellular component morphogenesis
51.	GO:0016043	2.43E-74	1285	cellular component organisation
52.	GO:0071840	1.96E-93	1369	cellular component organisation or biogenesis
53.	GO:0048869	2.00E-06	741	cellular developmental process
54.	GO:0051641	3.13E-09	502	cellular localisation
55.	GO:0034622	1.96E-27	212	cellular macromolecular complex assembly
56.	GO:0034645	2.06E-100	1234	cellular macromolecule biosynthetic process
57.	GO:0044265	1.36E-19	205	cellular macromolecule catabolic process
58.	GO:0070727	1.57E-10	254	cellular macromolecule localisation
59.	GO:0044260	4.00E-180	2021	cellular macromolecule metabolic process
60.	GO:0044237	3.53E-162	2446	cellular metabolic process
61.	GO:0044271	1.28E-45	966	cellular nitrogen compound biosynthetic process
62.	GO:0044270	2.11E-12	285	cellular nitrogen compound catabolic process
63.	GO:0034641	7.38E-131	1672	cellular nitrogen compound

				metabolic process
64.	GO:0009987	9.64E-65	3265	cellular process
65.	GO:0048610	3.82E-05	125	cellular process involved in reproduction
66.	GO:0044257	7.95E-08	108	cellular protein catabolic process
67.	GO:0043623	6.56E-11	117	cellular protein complex assembly
68.	GO:0043624	1.54E-05	36	cellular protein complex disassembly
69.	GO:0034613	4.24E-10	251	cellular protein localisation
70.	GO:0044267	1.83E-67	1016	cellular protein metabolic process
71.	GO:0006464	1.70E-24	700	cellular protein modification process
72.	GO:0045333	3.04E-06	38	cellular respiration
73.	GO:0006974	7.68E-42	261	cellular response to DNA damage stimulus
74.	GO:0071479	2.65E-05	21	cellular response to ionising radiation
75.	GO:0071478	3.70E-05	35	cellular response to radiation
76.	GO:0033554	7.62E-37	413	cellular response to stress
77.	GO:0034508	1.55E-07	15	centromere complex assembly
78.	GO:0007098	5.36E-09	27	centrosome cycle
79.	GO:0051298	5.34E-07	20	centrosome duplication
80.	GO:0051297	2.24E-15	45	centrosome organisation
81.	GO:0051299	1.95E-05	7	centrosome separation
82.	GO:0043009	3.34E-13	208	chordate embryonic development
83.	GO:0006333	4.17E-06	41	chromatin assembly or disassembly
84.	GO:0016568	1.61E-25	195	chromatin modification
85.	GO:0006325	1.03E-26	217	chromatin organisation
86.	GO:0006338	7.15E-06	35	chromatin remodeling
87.	GO:0030261	4.93E-05	16	chromosome condensation
88.	GO:0050000	1.37E-07	16	chromosome localisation
89.	GO:0051276	2.26E-44	306	chromosome organisation
90.	GO:0007059	4.95E-32	99	chromosome segregation
91.	GO:0072359	2.25E-10	221	circulatory system development
92.	GO:0030199	3.13E-08	24	collagen fibril organisation
93.	GO:0016569	4.57E-21	143	covalent chromatin modification
94.	GO:0010388	1.06E-06	9	cullin deneddylation
95.	GO:0016482	7.97E-12	197	cytoplasmic transport
96.	GO:0007010	3.78E-18	275	cytoskeleton organisation
97.	GO:0016265	3.07E-10	427	death
98.	GO:0032502	7.74E-17	1132	developmental process
99.	GO:0006308	1.64E-05	27	DNA catabolic process
100.	GO:0000737	8.53E-05	21	DNA catabolic process, endonucleolytic
101.	GO:0071103	1.39E-13	74	DNA conformation change
102.	GO:0000077	2.59E-08	40	DNA damage checkpoint
103.	GO:0000729	8.09E-05	6	DNA double-strand break processing
104.	GO:0032508	4.00E-09	23	DNA duplex unwinding
105.	GO:0032392	3.06E-09	24	DNA geometric change

106.	GO:0031570	8.97E-10	45	DNA integrity checkpoint
107.	GO:0006259	9.74E-65	374	DNA metabolic process
108.	GO:0006323	1.45E-07	49	DNA packaging
109.	GO:0006310	2.99E-17	89	DNA recombination
110.	GO:0006281	8.79E-44	194	DNA repair
111.	GO:0006260	3.49E-39	143	DNA replication
112.	GO:0006270	5.92E-07	13	DNA replication initiation
113.	GO:0006261	6.76E-19	55	DNA-dependent DNA replication
114.	GO:0006354	1.16E-05	22	DNA-templated transcription, elongation
115.	GO:0006352	1.35E-07	35	DNA-templated transcription, initiation
116.	GO:0006302	8.53E-18	66	double-strand break repair
117.	GO:0000724	6.51E-09	30	double-strand break repair via homologous recombination
118.	GO:0009790	7.01E-19	325	embryo development
119.	GO:0009792	1.30E-13	211	embryo development ending in birth or egg hatching
120.	GO:0035113	9.45E-05	44	embryonic appendage morphogenesis
121.	GO:0048701	3.93E-05	21	embryonic cranial skeleton morphogenesis
122.	GO:0001838	2.21E-05	44	embryonic epithelial tube formation
123.	GO:0030326	9.45E-05	44	embryonic limb morphogenesis
124.	GO:0048598	3.08E-09	170	embryonic morphogenesis
125.	GO:0048706	7.74E-08	53	embryonic skeletal system development
126.	GO:0048704	3.46E-06	40	embryonic skeletal system morphogenesis
127.	GO:0015980	2.01E-05	58	energy derivation by oxidation of organic compounds
128.	GO:0001837	2.20E-05	32	epithelial to mesenchymal transition
129.	GO:0072175	2.21E-05	44	epithelial tube formation
130.	GO:0060562	1.22E-06	97	epithelial tube morphogenesis
131.	GO:0006888	1.52E-07	25	ER to Golgi vesicle-mediated transport
132.	GO:0051303	3.08E-09	17	establishment of chromosome localisation
133.	GO:0051649	1.99E-05	409	establishment of localisation in cell
134.	GO:0045184	1.30E-06	320	establishment of protein localisation
135.	GO:0072594	3.41E-05	91	establishment of protein localisation to organelle
136.	GO:0051236	5.03E-26	71	establishment of RNA localisation
137.	GO:0030198	1.79E-05	59	extracellular matrix organisation
138.	GO:0043062	2.13E-05	59	extracellular structure organisation
139.	GO:0000082	1.73E-08	56	G1/S transition of mitotic cell cycle
140.	GO:0000086	3.51E-06	29	G2/M transition of mitotic cell cycle
141.	GO:0010467	2.01E-119	1587	gene expression
142.	GO:0016458	9.20E-05	41	gene silencing
143.	GO:0006091	8.34E-05	73	generation of precursor metabolites

				and energy
144.	GO:1901659	5.27E-05	34	glycosyl compound biosynthetic process
145.	GO:1901658	2.31E-05	189	glycosyl compound catabolic process
146.	GO:1901657	4.95E-08	237	glycosyl compound metabolic process
147.	GO:0048193	1.95E-05	47	Golgi vesicle transport
148.	GO:0007507	1.00E-11	146	heart development
149.	GO:0003007	1.68E-10	84	heart morphogenesis
150.	GO:0018130	1.04E-48	964	heterocycle biosynthetic process
151.	GO:0046700	6.83E-12	284	heterocycle catabolic process
152.	GO:0046483	3.09E-139	1660	heterocycle metabolic process
153.	GO:0016573	8.99E-09	51	histone acetylation
154.	GO:0043968	8.42E-05	9	histone H2A acetylation
155.	GO:0043966	1.19E-05	23	histone H3 acetylation
156.	GO:0043967	7.81E-06	23	histone H4 acetylation
157.	GO:0016571	6.07E-06	37	histone methylation
158.	GO:0016570	5.17E-21	141	histone modification
159.	GO:0016574	5.38E-05	17	histone ubiquitination
160.	GO:0001701	3.03E-09	133	in utero embryonic development
161.	GO:0018393	2.51E-08	51	internal peptidyl-lysine acetylation
162.	GO:0006475	3.23E-08	52	internal protein amino acid acetylation
163.	GO:0006886	3.63E-05	163	intracellular protein transport
164.	GO:0046907	1.91E-09	301	intracellular transport
165.	GO:0051382	1.00E-04	8	kinetochore assembly
166.	GO:0051383	8.28E-06	10	kinetochore organisation
167.	GO:0060173	1.21E-05	56	limb development
168.	GO:0035108	3.51E-06	54	limb morphogenesis
169.	GO:0065003	2.03E-27	361	macromolecular complex assembly
170.	GO:0032984	1.99E-05	43	macromolecular complex disassembly
171.	GO:0043933	2.02E-35	452	macromolecular complex subunit organisation
172.	GO:0009059	2.58E-95	1252	macromolecule biosynthetic process
173.	GO:0009057	1.30E-19	252	macromolecule catabolic process
174.	GO:0033036	8.72E-13	489	macromolecule localisation
175.	GO:0043170	8.25E-157	2167	macromolecule metabolic process
176.	GO:0043414	2.97E-07	63	macromolecule methylation
177.	GO:0043412	8.60E-28	737	macromolecule modification
178.	GO:0051651	8.82E-06	39	maintenance of location in cell
179.	GO:0045185	4.34E-05	38	maintenance of protein location
180.	GO:0032507	1.17E-05	36	maintenance of protein location in cell
181.	GO:0030490	2.83E-05	8	maturation of SSU-rRNA
182.	GO:0051321	1.76E-09	69	meiotic cell cycle
183.	GO:0007126	8.05E-09	65	meiotic nuclear division

184.	GO:0008152	2.42E-137	2674	metabolic process
185.	GO:0051310	9.87E-09	15	metaphase plate congression
186.	GO:0044784	1.23E-07	20	metaphase/anaphase transition of cell cycle
187.	GO:0007091	1.23E-07	20	metaphase/anaphase transition of mitotic cell cycle
188.	GO:0032259	5.72E-09	94	methylation
189.	GO:0034453	3.63E-05	18	microtubule anchoring
190.	GO:0000226	2.89E-22	140	microtubule cytoskeleton organisation
191.	GO:0031023	7.60E-15	46	microtubule organising center organisation
192.	GO:0007017	4.92E-19	176	microtubule-based process
193.	GO:0007005	6.18E-08	93	mitochondrion organisation
194.	GO:0007067	1.37E-50	195	mitosis
195.	GO:0000278	2.05E-58	302	mitotic cell cycle
196.	GO:0007093	8.50E-11	46	mitotic cell cycle checkpoint
197.	GO:0044772	3.08E-22	121	mitotic cell cycle phase transition
198.	GO:0007076	2.89E-05	9	mitotic chromosome condensation
199.	GO:0044774	8.53E-05	21	mitotic DNA integrity checkpoint
200.	GO:0007080	2.89E-05	9	mitotic metaphase plate congression
201.	GO:0000070	1.22E-14	35	mitotic sister chromatid segregation
202.	GO:0007094	1.21E-05	13	mitotic spindle assembly checkpoint
203.	GO:0071174	2.95E-06	15	mitotic spindle checkpoint
204.	GO:0007052	1.85E-07	21	mitotic spindle organisation
205.	GO:0043632	1.91E-11	125	modification-dependent macromolecule catabolic process
206.	GO:0019941	4.74E-11	123	modification-dependent protein catabolic process
207.	GO:0061138	7.18E-05	65	morphogenesis of a branching epithelium
208.	GO:0001763	7.72E-05	68	morphogenesis of a branching structure
209.	GO:0002009	9.61E-09	133	morphogenesis of an epithelium
210.	GO:0016331	5.38E-05	50	morphogenesis of embryonic epithelium
211.	GO:0006402	2.81E-07	38	mRNA catabolic process
212.	GO:0045292	1.95E-05	7	mRNA cis splicing, via spliceosome
213.	GO:0006406	9.42E-10	25	mRNA export from nucleus
214.	GO:0016071	8.32E-53	219	mRNA metabolic process
215.	GO:0006397	7.23E-54	201	mRNA processing
216.	GO:0000398	2.65E-34	87	mRNA splicing, via spliceosome
217.	GO:0051028	1.04E-24	62	mRNA transport
218.	GO:0007275	6.54E-12	978	multicellular organismal development
219.	GO:0055001	8.44E-05	48	muscle cell development
220.	GO:0042692	1.19E-05	91	muscle cell differentiation
221.	GO:0061061	2.00E-07	144	muscle structure development
222.	GO:0060537	2.78E-05	107	muscle tissue development

223.	GO:0034660	1.65E-36	151	ncRNA metabolic process
224.	GO:0034470	4.38E-28	115	ncRNA processing
225.	GO:0048519	2.14E-17	889	negative regulation of biological process
226.	GO:0009890	4.14E-15	326	negative regulation of biosynthetic process
227.	GO:0045786	1.10E-08	79	negative regulation of cell cycle
228.	GO:1901988	2.36E-12	58	negative regulation of cell cycle phase transition
229.	GO:0010948	3.70E-17	82	negative regulation of cell cycle process
230.	GO:0031327	6.48E-16	324	negative regulation of cellular biosynthetic process
231.	GO:0051129	4.71E-07	122	negative regulation of cellular component organisation
232.	GO:2000113	2.42E-17	308	negative regulation of cellular macromolecule biosynthetic process
233.	GO:0031324	7.01E-19	435	negative regulation of cellular metabolic process
234.	GO:0048523	5.46E-20	824	negative regulation of cellular process
235.	GO:0051053	2.03E-06	34	negative regulation of DNA metabolic process
236.	GO:0010629	1.08E-12	302	negative regulation of gene expression
237.	GO:0010558	2.83E-16	316	negative regulation of macromolecule biosynthetic process
238.	GO:0010605	3.78E-20	452	negative regulation of macromolecule metabolic process
239.	GO:0009892	1.62E-17	474	negative regulation of metabolic process
240.	GO:1902100	1.21E-06	15	negative regulation of metaphase/anaphase transition of cell cycle
241.	GO:0045839	7.55E-07	19	negative regulation of mitosis
242.	GO:1901991	9.17E-13	58	negative regulation of mitotic cell cycle phase transition
243.	GO:0045841	1.21E-06	15	negative regulation of mitotic metaphase/anaphase transition
244.	GO:0050686	3.92E-08	14	negative regulation of mRNA processing
245.	GO:0048025	6.81E-09	14	negative regulation of mRNA splicing, via spliceosome
246.	GO:0051172	9.75E-19	325	negative regulation of nitrogen compound metabolic process
247.	GO:0051784	4.06E-06	21	negative regulation of nuclear division
248.	GO:0045934	1.24E-19	323	negative regulation of nucleobase-containing compound metabolic process
249.	GO:0010639	8.73E-09	79	negative regulation of organelle

				organisation
250.	GO:0051248	9.56E-05	136	negative regulation of protein metabolic process
251.	GO:2001021	9.05E-06	19	negative regulation of response to DNA damage stimulus
252.	GO:1902679	6.73E-13	268	negative regulation of RNA biosynthetic process
253.	GO:0051253	9.44E-16	286	negative regulation of RNA metabolic process
254.	GO:0033119	4.33E-08	16	negative regulation of RNA splicing
255.	GO:0000122	2.31E-09	180	negative regulation of transcription from RNA polymerase II promoter
256.	GO:0045892	8.57E-13	263	negative regulation of transcription, DNA-templated
257.	GO:0017148	8.78E-05	29	negative regulation of translation
258.	GO:0001843	4.99E-05	32	neural tube closure
259.	GO:0001841	4.34E-05	38	neural tube formation
260.	GO:0006807	4.18E-126	1731	nitrogen compound metabolic process
261.	GO:0000726	4.87E-05	11	non-recombinational repair
262.	GO:0000280	8.19E-51	241	nuclear division
263.	GO:0051168	1.87E-13	53	nuclear export
264.	GO:0051170	8.36E-06	72	nuclear import
265.	GO:0006999	4.64E-06	8	nuclear pore organisation
266.	GO:0051169	3.02E-16	132	nuclear transport
267.	GO:0000956	1.00E-05	30	nuclear-transcribed mRNA catabolic process
268.	GO:0000184	5.25E-06	19	nuclear-transcribed mRNA catabolic process, nonsense-mediated decay
269.	GO:0090304	1.70E-139	1394	nucleic acid metabolic process
270.	GO:0090305	4.36E-08	56	nucleic acid phosphodiester bond hydrolysis
271.	GO:0050657	5.03E-26	71	nucleic acid transport
272.	GO:0034654	1.14E-47	945	nucleobase-containing compound biosynthetic process
273.	GO:0034655	3.55E-13	280	nucleobase-containing compound catabolic process
274.	GO:0006139	1.35E-140	1632	nucleobase-containing compound metabolic process
275.	GO:0015931	1.88E-20	75	nucleobase-containing compound transport
276.	GO:0055086	9.12E-09	307	nucleobase-containing small molecule metabolic process
277.	GO:0006913	6.61E-16	130	nucleocytoplasmic transport
278.	GO:0009163	4.06E-05	34	nucleoside biosynthetic process
279.	GO:0009164	1.39E-05	189	nucleoside catabolic process
280.	GO:0009116	1.21E-08	237	nucleoside metabolic process
281.	GO:0009124	1.38E-05	30	nucleoside monophosphate biosynthetic process
282.	GO:0009125	2.60E-07	70	nucleoside monophosphate

				catabolic process
283.	GO:0009123	1.70E-10	98	nucleoside monophosphate metabolic process
284.	GO:1901292	4.34E-07	212	nucleoside phosphate catabolic process
285.	GO:0006753	1.31E-08	297	nucleoside phosphate metabolic process
286.	GO:0009143	1.24E-06	193	nucleoside triphosphate catabolic process
287.	GO:0009141	1.03E-07	212	nucleoside triphosphate metabolic process
288.	GO:0034728	4.06E-05	34	nucleosome organisation
289.	GO:0009166	1.37E-06	207	nucleotide catabolic process
290.	GO:0009117	3.04E-08	293	nucleotide metabolic process
291.	GO:0006997	7.15E-06	30	nucleus organisation
292.	GO:0048513	3.34E-05	559	organ development
293.	GO:0009887	2.60E-07	227	organ morphogenesis
294.	GO:0070925	2.73E-11	111	organelle assembly
295.	GO:0048285	3.13E-50	249	organelle fission
296.	GO:0006996	1.25E-83	865	organelle organisation
297.	GO:1901362	1.60E-48	990	organic cyclic compound biosynthetic process
298.	GO:1901361	5.45E-11	288	organic cyclic compound catabolic process
299.	GO:1901360	1.21E-133	1697	organic cyclic compound metabolic process
300.	GO:1901576	1.78E-92	1432	organic substance biosynthetic process
301.	GO:1901575	3.83E-14	517	organic substance catabolic process
302.	GO:0071704	1.17E-149	2537	organic substance metabolic process
303.	GO:1901564	6.09E-06	421	organonitrogen compound metabolic process
304.	GO:0046434	1.59E-06	221	organophosphate catabolic process
305.	GO:0019637	3.25E-05	337	organophosphate metabolic process
306.	GO:0003151	2.51E-05	25	outflow tract morphogenesis
307.	GO:0060021	2.66E-05	33	palate development
308.	GO:0018193	5.66E-10	216	peptidyl-amino acid modification
309.	GO:0018196	6.53E-05	10	peptidyl-asparagine modification
310.	GO:0018394	4.46E-08	52	peptidyl-lysine acetylation
311.	GO:0018205	1.76E-08	68	peptidyl-lysine modification
312.	GO:0018208	1.66E-08	21	peptidyl-proline modification
313.	GO:0006796	8.99E-10	731	phosphate-containing compound metabolic process
314.	GO:0006793	5.16E-10	743	phosphorus metabolic process
315.	GO:0048518	9.83E-13	939	positive regulation of biological process
316.	GO:0009891	2.36E-16	405	positive regulation of biosynthetic process

317.	GO:0090263	2.42E-05	26	positive regulation of canonical Wnt signaling pathway
318.	GO:1901989	1.74E-05	21	positive regulation of cell cycle phase transition
319.	GO:0090068	6.78E-07	60	positive regulation of cell cycle process
320.	GO:0031328	3.82E-17	401	positive regulation of cellular biosynthetic process
321.	GO:0051130	5.32E-06	182	positive regulation of cellular component organisation
322.	GO:0031325	2.97E-15	550	positive regulation of cellular metabolic process
323.	GO:0048522	3.89E-19	885	positive regulation of cellular process
324.	GO:2001252	5.21E-05	24	positive regulation of chromosome organisation
325.	GO:0051054	1.13E-05	44	positive regulation of DNA metabolic process
326.	GO:2000105	8.09E-05	6	positive regulation of DNA-dependent DNA replication
327.	GO:0010628	6.35E-15	356	positive regulation of gene expression
328.	GO:0010557	1.70E-17	380	positive regulation of macromolecule biosynthetic process
329.	GO:0010604	2.63E-16	536	positive regulation of macromolecule metabolic process
330.	GO:0009893	2.08E-14	577	positive regulation of metabolic process
331.	GO:1901992	1.74E-05	21	positive regulation of mitotic cell cycle phase transition
332.	GO:0051173	5.01E-17	387	positive regulation of nitrogen compound metabolic process
333.	GO:0045935	8.53E-18	383	positive regulation of nucleobase-containing compound metabolic process
334.	GO:1902680	2.97E-16	336	positive regulation of RNA biosynthetic process
335.	GO:0051254	5.51E-17	344	positive regulation of RNA metabolic process
336.	GO:0045944	8.35E-09	230	positive regulation of transcription from RNA polymerase II promoter
337.	GO:0045893	2.43E-15	329	positive regulation of transcription, DNA-templated
338.	GO:0045727	8.12E-06	26	positive regulation of translation
339.	GO:0030177	4.99E-05	32	positive regulation of Wnt signaling pathway
340.	GO:0010608	1.22E-15	153	posttranscriptional regulation of gene expression
341.	GO:0044238	1.59E-157	2476	primary metabolic process
342.	GO:0014020	3.18E-05	34	primary neural tube formation
343.	GO:0012501	2.47E-09	402	programmed cell death
344.	GO:0006473	1.61E-08	57	protein acetylation

345.	GO:0043543	2.87E-08	66	protein acylation
346.	GO:0008213	2.50E-05	43	protein alkylation
347.	GO:0030163	2.07E-12	175	protein catabolic process
348.	GO:0006461	4.56E-17	294	protein complex assembly
349.	GO:0070271	3.26E-15	240	protein complex biogenesis
350.	GO:0043241	4.51E-06	43	protein complex disassembly
351.	GO:0031503	8.72E-05	20	protein complex localisation
352.	GO:0071822	2.55E-25	383	protein complex subunit organisation
353.	GO:0000338	1.06E-06	9	protein deneddylation
354.	GO:0051261	3.18E-05	31	protein depolymerisation
355.	GO:0006457	1.26E-17	75	protein folding
356.	GO:0017038	3.25E-05	76	protein import
357.	GO:0006606	1.13E-05	71	protein import into nucleus
358.	GO:0008104	5.33E-12	436	protein localisation
359.	GO:0034502	6.88E-07	17	protein localisation to chromosome
360.	GO:0034501	1.95E-05	7	protein localisation to kinetochore
361.	GO:0034504	1.20E-06	87	protein localisation to nucleus
362.	GO:0033365	4.80E-11	146	protein localisation to organelle
363.	GO:0019538	2.87E-53	1160	protein metabolic process
364.	GO:0006479	2.50E-05	43	protein methylation
365.	GO:0032446	1.66E-09	144	protein modification by small protein conjugation
366.	GO:0070647	4.81E-15	183	protein modification by small protein conjugation or removal
367.	GO:0070646	5.72E-08	41	protein modification by small protein removal
368.	GO:0036211	1.70E-24	700	protein modification process
369.	GO:0006513	3.93E-05	21	protein monoubiquitination
370.	GO:0000413	1.21E-06	15	protein peptidyl-prolyl isomerisation
371.	GO:0000209	8.92E-06	46	protein polyubiquitination
372.	GO:0015031	6.01E-06	299	protein transport
373.	GO:0016567	6.99E-09	133	protein ubiquitination
374.	GO:0065004	4.57E-07	42	protein-DNA complex assembly
375.	GO:0071824	1.91E-09	54	protein-DNA complex subunit organisation
376.	GO:0051603	2.03E-11	136	proteolysis involved in cellular protein catabolic process
377.	GO:0006152	8.29E-06	189	purine nucleoside catabolic process
378.	GO:0042278	3.62E-07	218	purine nucleoside metabolic process
379.	GO:0009128	3.65E-07	69	purine nucleoside monophosphate catabolic process
380.	GO:0009126	4.81E-09	90	purine nucleoside monophosphate metabolic process
381.	GO:0009146	3.93E-06	189	purine nucleoside triphosphate catabolic process
382.	GO:0009144	7.17E-07	204	purine nucleoside triphosphate metabolic process
383.	GO:0006195	4.25E-07	206	purine nucleotide catabolic process

384.	GO:0006163	1.94E-07	257	purine nucleotide metabolic process
385.	GO:0046130	8.29E-06	189	purine ribonucleoside catabolic process
386.	GO:0046128	3.65E-07	217	purine ribonucleoside metabolic process
387.	GO:0009169	2.92E-07	69	purine ribonucleoside monophosphate catabolic process
388.	GO:0009167	3.81E-09	90	purine ribonucleoside monophosphate metabolic process
389.	GO:0009207	2.96E-06	189	purine ribonucleoside triphosphate catabolic process
390.	GO:0009205	5.93E-07	203	purine ribonucleoside triphosphate metabolic process
391.	GO:0009154	2.44E-06	195	purine ribonucleotide catabolic process
392.	GO:0009150	1.51E-06	243	purine ribonucleotide metabolic process
393.	GO:0072523	5.69E-07	207	purine-containing compound catabolic process
394.	GO:0072521	2.39E-07	266	purine-containing compound metabolic process
395.	GO:0006221	9.33E-05	13	pyrimidine nucleotide biosynthetic process
396.	GO:0000725	6.51E-09	30	recombinational repair
397.	GO:0000381	2.91E-05	16	regulation of alternative mRNA splicing, via spliceosome
398.	GO:0022603	2.91E-05	185	regulation of anatomical structure morphogenesis
399.	GO:0042981	1.10E-07	306	regulation of apoptotic process
400.	GO:0009889	9.36E-44	979	regulation of biosynthetic process
401.	GO:0060828	1.32E-05	52	regulation of canonical Wnt signaling pathway
402.	GO:0051726	2.29E-23	236	regulation of cell cycle
403.	GO:0071156	8.72E-05	20	regulation of cell cycle arrest
404.	GO:1901976	2.57E-05	13	regulation of cell cycle checkpoint
405.	GO:1901987	3.74E-16	87	regulation of cell cycle phase transition
406.	GO:0010564	9.02E-23	159	regulation of cell cycle process
407.	GO:0010941	1.65E-08	329	regulation of cell death
408.	GO:0051302	1.63E-07	72	regulation of cell division
409.	GO:0022604	6.28E-05	101	regulation of cell morphogenesis
410.	GO:0042127	1.41E-05	308	regulation of cell proliferation
411.	GO:0031326	7.72E-46	971	regulation of cellular biosynthetic process
412.	GO:0051128	2.06E-11	412	regulation of cellular component organisation
413.	GO:2000112	8.44E-51	935	regulation of cellular macromolecule biosynthetic process
414.	GO:0031323	8.44E-51	1286	regulation of cellular metabolic process

415.	GO:0032268	1.80E-11	363	regulation of cellular protein metabolic process
416.	GO:0080135	9.05E-06	103	regulation of cellular response to stress
417.	GO:0046605	5.00E-05	13	regulation of centrosome cycle
418.	GO:0033044	8.77E-07	51	regulation of chromosome organisation
419.	GO:0051983	5.08E-07	18	regulation of chromosome segregation
420.	GO:0051493	3.21E-06	98	regulation of cytoskeleton organisation
421.	GO:0050793	6.09E-06	428	regulation of developmental process
422.	GO:0051052	9.88E-14	103	regulation of DNA metabolic process
423.	GO:0000018	7.87E-05	23	regulation of DNA recombination
424.	GO:0006282	5.81E-07	27	regulation of DNA repair
425.	GO:0006275	1.41E-07	45	regulation of DNA replication
426.	GO:0090329	6.40E-06	15	regulation of DNA-dependent DNA replication
427.	GO:2000779	1.84E-05	14	regulation of double-strand break repair
428.	GO:2000045	3.82E-05	32	regulation of G1/S transition of mitotic cell cycle
429.	GO:0010468	8.97E-46	1035	regulation of gene expression
430.	GO:0040029	3.49E-05	55	regulation of gene expression, epigenetic
431.	GO:0010556	7.37E-47	948	regulation of macromolecule biosynthetic process
432.	GO:0060255	1.73E-50	1228	regulation of macromolecule metabolic process
433.	GO:0019222	4.41E-43	1404	regulation of metabolic process
434.	GO:1902099	2.60E-07	20	regulation of metaphase/anaphase transition of cell cycle
435.	GO:0070507	1.05E-08	47	regulation of microtubule cytoskeleton organisation
436.	GO:0032886	1.00E-07	50	regulation of microtubule-based process
437.	GO:0007088	2.21E-06	38	regulation of mitosis
438.	GO:0007346	2.04E-17	125	regulation of mitotic cell cycle
439.	GO:1901990	1.63E-16	87	regulation of mitotic cell cycle phase transition
440.	GO:0030071	2.60E-07	20	regulation of mitotic metaphase/anaphase transition
441.	GO:0050684	3.85E-12	39	regulation of mRNA processing
442.	GO:0048024	1.55E-11	30	regulation of mRNA splicing, via spliceosome
443.	GO:0051171	6.71E-48	1034	regulation of nitrogen compound metabolic process
444.	GO:0051783	1.94E-06	46	regulation of nuclear division
445.	GO:0019219	2.29E-49	1027	regulation of nucleobase-containing compound metabolic process

446.	GO:0046822	1.22E-05	59	regulation of nucleocytoplasmic transport
447.	GO:0033043	2.67E-14	212	regulation of organelle organisation
448.	GO:0080090	1.39E-51	1263	regulation of primary metabolic process
449.	GO:0043067	1.02E-07	310	regulation of programmed cell death
450.	GO:0042176	6.19E-06	66	regulation of protein catabolic process
451.	GO:0051246	3.11E-09	399	regulation of protein metabolic process
452.	GO:2001020	4.14E-10	52	regulation of response to DNA damage stimulus
453.	GO:2001141	7.31E-38	831	regulation of RNA biosynthetic process
454.	GO:0051252	4.18E-43	867	regulation of RNA metabolic process
455.	GO:0043484	2.40E-11	41	regulation of RNA splicing
456.	GO:0006357	9.38E-16	406	regulation of transcription from RNA polymerase II promoter
457.	GO:0006355	5.50E-37	824	regulation of transcription, DNA-templated
458.	GO:0006417	1.98E-17	99	regulation of translation
459.	GO:0006446	1.05E-08	27	regulation of translational initiation
460.	GO:0030111	3.18E-05	64	regulation of Wnt signaling pathway
461.	GO:0010212	5.82E-07	43	response to ionising radiation
462.	GO:0009314	8.10E-05	87	response to radiation
463.	GO:0006950	2.05E-08	600	response to stress
464.	GO:0009411	3.90E-05	39	response to UV
465.	GO:0022618	3.22E-19	62	ribonucleoprotein complex assembly
466.	GO:0022613	6.22E-52	154	ribonucleoprotein complex biogenesis
467.	GO:0071826	3.95E-17	62	ribonucleoprotein complex subunit organisation
468.	GO:0042455	4.06E-05	34	ribonucleoside biosynthetic process
469.	GO:0042454	1.18E-05	189	ribonucleoside catabolic process
470.	GO:0009119	1.24E-07	223	ribonucleoside metabolic process
471.	GO:0009156	8.69E-05	26	ribonucleoside monophosphate biosynthetic process
472.	GO:0009158	2.92E-07	69	ribonucleoside monophosphate catabolic process
473.	GO:0009161	1.91E-09	92	ribonucleoside monophosphate metabolic process
474.	GO:0009203	2.16E-06	190	ribonucleoside triphosphate catabolic process
475.	GO:0009199	2.29E-07	206	ribonucleoside triphosphate metabolic process
476.	GO:0009261	2.44E-06	195	ribonucleotide catabolic process
477.	GO:0009259	6.88E-07	247	ribonucleotide metabolic process
478.	GO:0019693	7.37E-07	248	ribose phosphate metabolic process

479.	GO:0042273	5.81E-07	12	ribosomal large subunit biogenesis
480.	GO:0000028	1.06E-06	9	ribosomal small subunit assembly
481.	GO:0042274	8.43E-12	20	ribosomal small subunit biogenesis
482.	GO:0042255	1.55E-07	15	ribosome assembly
483.	GO:0042254	3.41E-42	113	ribosome biogenesis
484.	GO:0031123	3.37E-05	26	RNA 3'-end processing
485.	GO:0032774	3.17E-43	863	RNA biosynthetic process
486.	GO:0006401	7.08E-09	48	RNA catabolic process
487.	GO:0006405	1.42E-12	32	RNA export from nucleus
488.	GO:0006403	2.62E-24	70	RNA localisation
489.	GO:0016070	1.56E-98	1169	RNA metabolic process
490.	GO:0009451	1.15E-05	27	RNA modification
491.	GO:0006396	5.73E-81	322	RNA processing
492.	GO:0008380	6.06E-52	169	RNA splicing
493.	GO:0000375	7.48E-35	88	RNA splicing, via transesterification reactions
494.	GO:0000377	2.65E-34	87	RNA splicing, via transesterification reactions with bulged adenosine as nucleophile
495.	GO:0050658	5.03E-26	71	RNA transport
496.	GO:0016072	9.88E-27	77	rRNA metabolic process
497.	GO:0006364	4.46E-25	73	rRNA processing
498.	GO:0009303	4.87E-05	11	rRNA transcription
499.	GO:0044711	8.56E-05	276	single-organism biosynthetic process
500.	GO:0044763	2.13E-05	2429	single-organism cellular process
501.	GO:0044767	1.17E-16	1127	single-organism developmental process
502.	GO:1902582	1.02E-06	236	single-organism intracellular transport
503.	GO:0044710	4.07E-26	1150	single-organism metabolic process
504.	GO:1902593	1.13E-05	71	single-organism nuclear import
505.	GO:1902589	1.89E-67	622	single-organism organelle organisation
506.	GO:0044699	3.21E-08	2738	single-organism process
507.	GO:0000819	4.00E-15	40	sister chromatid segregation
508.	GO:0001501	3.29E-12	137	skeletal system development
509.	GO:0048705	1.42E-07	75	skeletal system morphogenesis
510.	GO:0044281	2.31E-09	539	small molecule metabolic process
511.	GO:0051225	7.89E-13	30	spindle assembly
512.	GO:0071173	3.73E-07	16	spindle assembly checkpoint
513.	GO:0031577	3.95E-08	21	spindle checkpoint
514.	GO:0007051	3.74E-20	52	spindle organisation
515.	GO:0000245	8.93E-06	16	spliceosomal complex assembly
516.	GO:0000387	1.98E-10	19	spliceosomal snRNP assembly
517.	GO:0002040	8.72E-05	20	sprouting angiogenesis
518.	GO:0016126	1.16E-05	22	sterol biosynthetic process
519.	GO:0014706	3.92E-05	102	striated muscle tissue development
520.	GO:0048731	5.56E-08	781	system development

521.	GO:0009888	2.60E-07	354	tissue development
522.	GO:0048729	3.20E-09	164	tissue morphogenesis
523.	GO:0006366	6.85E-17	416	transcription from RNA polymerase II promoter
524.	GO:0006367	3.91E-05	22	transcription initiation from RNA polymerase II promoter
525.	GO:0006351	2.57E-42	855	transcription, DNA-templated
526.	GO:0006412	2.96E-61	243	translation
527.	GO:0006414	1.52E-08	26	translational elongation
528.	GO:0006413	2.14E-18	54	translational initiation
529.	GO:0043039	2.76E-10	29	tRNA aminoacylation
530.	GO:0006418	2.01E-11	29	tRNA aminoacylation for protein translation
531.	GO:0006399	9.43E-12	60	tRNA metabolic process
532.	GO:0030488	8.09E-05	6	tRNA methylation
533.	GO:0006400	6.40E-06	15	tRNA modification
534.	GO:0060606	6.53E-05	32	tube closure
535.	GO:0035295	2.55E-07	140	tube development
536.	GO:0035148	4.97E-05	46	tube formation
537.	GO:0035239	7.61E-06	98	tube morphogenesis
538.	GO:0006511	2.75E-11	122	ubiquitin-dependent protein catabolic process
539.	GO:0016055	3.51E-05	100	Wnt signaling pathway

Table S3 Biological processes (BP) revealed by gene ontology (GO) analysis associated with upregulated genes upon HP1 γ knockout in female MEFs. The number of 'count' represent the number of dysregulated genes contribute to each term. Adjusted P-value <10⁻⁵.

	GOBPID	Adjusted P-value	Count	Term
1.	GO:0022610	1.39E-07	61	biological adhesion
2.	GO:0007155	1.39E-07	61	cell adhesion
3.	GO:0000904	9.72E-05	38	cell morphogenesis involved in differentiation
4.	GO:0006749	1.13E-05	9	glutathione metabolic process
5.	GO:0007399	9.72E-05	81	nervous system development

Table S4 Biological processes (BP) revealed by gene ontology (GO) analysis associated with downregulated genes upon HP1 γ knockout in female MEFs. The number of 'count' represent the number of dyregulated genes contribute to each term. Adjusted P-value <10⁻⁵.

	GOBPID	Adjusted P-value	Count	Term
1.	GO:0009653	1.65E-05	73	anatomical structure morphogenesis
2.	GO:0009952	1.10E-05	17	anterior/posterior pattern specification
3.	GO:0072358	7.61E-05	32	cardiovascular system development
4.	GO:0051216	5.30E-05	13	cartilage development
5.	GO:0007049	1.65E-05	50	cell cycle
6.	GO:0022402	3.56E-06	43	cell cycle process
7.	GO:0016043	5.00E-06	129	cellular component organisation
8.	GO:0071840	1.10E-05	129	cellular component organisation or biogenesis
9.	GO:0044260	1.56E-05	169	cellular macromolecule metabolic process
10.	GO:0044237	7.58E-06	219	cellular metabolic process
11.	GO:0009987	3.74E-05	325	cellular process
12.	GO:0006464	2.76E-05	81	cellular protein modification process
13.	GO:0007059	9.20E-05	12	chromosome segregation
14.	GO:0072359	7.61E-05	32	circulatory system development
15.	GO:0007010	7.61E-05	35	cytoskeleton organisation
16.	GO:0032502	8.49E-05	128	developmental process
17.	GO:0009790	3.13E-05	42	embryo development
18.	GO:0048598	1.56E-05	29	embryonic morphogenesis
19.	GO:0060429	5.43E-05	32	epithelium development
20.	GO:0051234	8.49E-05	106	establishment of localisation
21.	GO:0007507	1.32E-05	25	heart development
22.	GO:0035404	2.76E-05	4	histone-serine phosphorylation
23.	GO:0051179	3.56E-06	139	localisation
24.	GO:0033036	9.66E-05	61	macromolecule localisation
25.	GO:0043170	3.74E-05	185	macromolecule metabolic process
26.	GO:0043412	7.38E-05	81	macromolecule modification
27.	GO:0048762	9.20E-05	10	mesenchymal cell differentiation
28.	GO:0008152	5.11E-05	240	metabolic process
29.	GO:0007067	7.83E-06	23	mitosis
30.	GO:0000278	2.76E-05	30	mitotic cell cycle
31.	GO:0048519	6.61E-05	103	negative regulation of biological process
32.	GO:0048523	7.61E-05	94	negative regulation of cellular process
33.	GO:0006807	9.42E-05	147	nitrogen compound metabolic process
34.	GO:0071705	1.10E-05	30	nitrogen compound transport
35.	GO:0000280	1.65E-05	26	nuclear division
36.	GO:0048513	1.56E-05	81	organ development
37.	GO:0009887	1.93E-05	37	organ morphogenesis

38.	GO:0048285	1.10E-05	28	organelle fission
39.	GO:0006996	1.10E-05	82	organelle organisation
40.	GO:0071704	1.92E-05	226	organic substance metabolic process
41.	GO:0071702	1.56E-05	68	organic substance transport
42.	GO:0001503	5.42E-05	19	ossification
43.	GO:0007389	5.00E-06	27	pattern specification process
44.	GO:0006796	1.10E-05	97	phosphate-containing compound metabolic process
45.	GO:0006793	1.56E-05	97	phosphorus metabolic process
46.	GO:0016310	9.66E-05	58	phosphorylation
47.	GO:0048518	1.65E-05	116	positive regulation of biological process
48.	GO:0031325	7.61E-05	67	positive regulation of cellular metabolic process
49.	GO:0048522	1.84E-05	105	positive regulation of cellular process
50.	GO:0032270	7.96E-05	33	positive regulation of cellular protein metabolic process
51.	GO:0010604	7.61E-05	65	positive regulation of macromolecule metabolic process
52.	GO:0009893	2.77E-05	73	positive regulation of metabolic process
53.	GO:0051247	3.74E-05	37	positive regulation of protein metabolic process
54.	GO:0044238	2.57E-05	217	primary metabolic process
55.	GO:0036211	2.76E-05	81	protein modification process
56.	GO:0016567	6.89E-05	22	protein ubiquitination
57.	GO:0003002	5.43E-05	19	regionalisation
58.	GO:0010646	7.61E-05	74	regulation of cell communication
59.	GO:0031323	7.61E-05	127	regulation of cellular metabolic process
60.	GO:0046599	3.74E-05	4	regulation of centriole replication
61.	GO:0046605	7.61E-05	5	regulation of centrosome cycle
62.	GO:0010824	3.74E-05	5	regulation of centrosome duplication
63.	GO:0032879	1.84E-05	63	regulation of localisation
64.	GO:0019222	1.84E-05	147	regulation of metabolic process
65.	GO:0080090	8.49E-05	124	regulation of primary metabolic process
66.	GO:0023051	7.61E-05	74	regulation of signaling
67.	GO:0044767	7.61E-05	128	single-organism developmental process
68.	GO:1902589	1.10E-05	62	single-organism organelle organisation
69.	GO:0001501	1.65E-05	23	skeletal system development
70.	GO:0009888	3.56E-06	58	tissue development

Table S5 List of PANTHER GO-Slim biological processes revealed by PANTHER overrepresentation test associated with upregulated genes upon HP1 γ knockout in male MEFs. Over- or underrepresentation groups with P<0.05 are presented. Definition of the PANTHER GO-Slim biological process can be found on the World Wide Web site for PANTHER classification system at: <http://www.pantherdb.org/>

	PANTHER GO-Slim Biological Process	No. of genes in mouse ref. list	No. of up-regulated genes in male	No. of expected genes	Over(+)/under(-) representation	Fold enrichment	P-value
1.	response to interferon-gamma (GO:0034341)	48	18	4.99	+	3.61	1.13E-03
2.	antigen processing and presentation (GO:0019882)	77	27	8.01	+	3.37	2.16E-05
3.	cell proliferation (GO:0008283)	110	28	11.44	+	2.45	5.18E-03
4.	B cell mediated immunity (GO:0019724)	155	39	16.12	+	2.42	1.95E-04
5.	cellular defense response (GO:0006968)	238	59	24.75	+	2.38	6.18E-07
6.	macrophage activation (GO:0042116)	173	41	17.99	+	2.28	4.48E-04
7.	immune response (GO:0006955)	537	116	55.84	+	2.08	1.38E-10
8.	phospholipid metabolic process (GO:0006644)	177	37	18.41	+	2.01	1.83E-02
9.	cell-cell adhesion (GO:0016337)	362	75	37.64	+	1.99	8.65E-06
10.	polysaccharide metabolic process (GO:0005976)	186	38	19.34	+	1.96	2.34E-02
11.	endocytosis (GO:0006897)	380	77	39.52	+	1.95	1.38E-05
12.	immune system process (GO:0002376)	1480	295	153.9	+	1.92	8.04E-24
13.	receptor-mediated endocytosis (GO:0006898)	217	43	22.57	+	1.91	1.68E-02
14.	visual perception (GO:0007601)	223	43	23.19	+	1.85	3.00E-02
15.	response to external stimulus (GO:0009605)	404	76	42.01	+	1.81	2.65E-04
16.	cell adhesion (GO:0007155)	550	102	57.19	+	1.78	8.38E-06
17.	biological adhesion (GO:0022610)	577	107	60	+	1.78	3.91E-06
18.	vesicle-mediated transport (GO:0016192)	891	147	92.65	+	1.59	1.33E-05
19.	phosphate-containing	931	152	96.81	+	1.57	1.47E-05

	compound metabolic process (GO:0006796)						
20.	lipid metabolic process (GO:0006629)	966	156	100.45	+	1.55	1.90E-05
21.	ion transport (GO:0006811)	723	114	75.18	+	1.52	2.92E-03
22.	cation transport (GO:0006812)	579	90	60.21	+	1.49	3.64E-02
23.	cell communication (GO:0007154)	3175	492	330.16	+	1.49	1.50E-17
24.	response to stimulus (GO:0050896)	2559	382	266.1	+	1.44	1.15E-10
25.	regulation of molecular function (GO:0065009)	1152	171	119.79	+	1.43	7.54E-04
26.	nervous system development (GO:0007399)	782	115	81.32	+	1.41	4.24E-02
27.	regulation of catalytic activity (GO:0050790)	1129	166	117.4	+	1.41	1.75E-03
28.	localization (GO:0051179)	2788	401	289.92	+	1.38	3.85E-09
29.	transport (GO:0006810)	2658	379	276.4	+	1.37	4.55E-08
30.	cellular protein modification process (GO:0006464)	1341	191	139.45	+	1.37	2.37E-03
31.	intracellular protein transport (GO:0006886)	1034	146	107.52	+	1.36	3.79E-02
32.	system development (GO:0048731)	1273	178	132.38	+	1.34	1.24E-02
33.	developmental process (GO:0032502)	2468	319	256.64	+	1.24	7.88E-03
34.	cellular process (GO:0009987)	7033	885	731.34	+	1.21	1.84E-09
35.	metabolic process (GO:0008152)	8467	1031	880.46	+	1.17	2.23E-08
36.	RNA metabolic process (GO:0016070)	2315	183	240.73	-	0.76	5.92E-03
37.	Unclassified (UNCLASSIFIED)	9561	745	994.22	-	0.75	0.00E+00
38.	DNA metabolic process (GO:0006259)	352	17	36.6	-	0.46	4.94E-02
39.	translation (GO:0006412)	479	22	49.81	-	0.44	1.48E-03
40.	sensory perception of chemical stimulus (GO:0007606)	295	5	30.68	-	< 0.2	2.44E-06
41.	rRNA metabolic process (GO:0016072)	120	1	12.48	-	< 0.2	1.10E-02

Table S6 List of PANTHER GO-Slim biological processes revealed by PANTHER overrepresentation test associated with downregulated genes upon HP1 γ knockout in male MEFs. Over- or underrepresentation groups with P<0.05 are presented. Definition of the PANTHER GO-Slim biological process can be found on the World Wide Web site for PANTHER classification system at: <http://www.pantherdb.org/>

	PANTHER GO-Slim Biological Process	No. of genes in mouse ref. list	No. of up-regulated genes in male	No. of expected genes	Over(+)/under(-) representation	Fold enrichment	P-value
1.	regulation of cell cycle (GO:0051726)	27	13	1.87	+	6.94	2.11E-05
2.	DNA recombination (GO:0006310)	50	18	3.47	+	5.19	6.47E-06
3.	DNA replication (GO:0006260)	152	49	10.54	+	4.65	1.00E-15
4.	regulation of liquid surface tension (GO:0050828)	55	16	3.81	+	4.19	5.74E-04
5.	chromosome segregation (GO:0007059)	84	24	5.83	+	4.12	2.88E-06
6.	DNA repair (GO:0006281)	168	46	11.65	+	3.95	3.59E-12
7.	DNA metabolic process (GO:0006259)	352	85	24.41	+	3.48	7.23E-20
8.	tRNA metabolic process (GO:0006399)	81	18	5.62	+	3.2	5.23E-03
9.	mRNA splicing, via spliceosome (GO:0000398)	187	41	12.97	+	3.16	7.35E-08
10.	rRNA metabolic process (GO:0016072)	120	26	8.32	+	3.12	1.46E-04
11.	cell-matrix adhesion (GO:0007160)	88	18	6.1	+	2.95	1.49E-02
12.	RNA splicing, via transesterification reactions (GO:0000375)	133	26	9.22	+	2.82	9.12E-04
13.	RNA splicing (GO:0008380)	136	26	9.43	+	2.76	1.34E-03
14.	chromatin organization (GO:0006325)	244	44	16.92	+	2.6	5.27E-06
15.	mitosis (GO:0007067)	365	63	25.31	+	2.49	3.02E-08
16.	mRNA processing (GO:0006397)	272	45	18.86	+	2.39	4.04E-05
17.	cell-cell adhesion (GO:0016337)	362	54	25.11	+	2.15	6.41E-05
18.	cell cycle (GO:0007049)	1128	160	78.23	+	2.05	8.29E-15
19.	cellular component biogenesis	298	39	20.67	+	1.89	4.10E-02

	(GO:0044085)						
20.	biological adhesion (GO:0022610)	577	73	40.02	+	1.82	2.83E-04
21.	ectoderm development (GO:0007398)	621	78	43.07	+	1.81	1.61E-04
22.	cell adhesion (GO:0007155)	550	69	38.15	+	1.81	7.24E-04
23.	translation (GO:0006412)	479	59	33.22	+	1.78	6.06E-03
24.	organelle organization (GO:0006996)	565	69	39.19	+	1.76	1.73E-03
25.	cellular component movement (GO:0006928)	463	55	32.11	+	1.71	2.77E-02
26.	cellular component organization or biogenesis (GO:0071840)	1298	154	90.02	+	1.71	3.02E-08
27.	nervous system development (GO:0007399)	782	90	54.24	+	1.66	7.93E-04
28.	nucleobase-containing compound metabolic process (GO:0006139)	3425	390	237.54	+	1.64	1.86E-21
29.	anatomical structure morphogenesis (GO:0009653)	584	65	40.5	+	1.6	4.32E-02
30.	nitrogen compound metabolic process (GO:0006807)	1064	117	73.79	+	1.59	2.47E-04
31.	cellular component organization (GO:0016043)	1185	130	82.19	+	1.58	6.90E-05
32.	system development (GO:0048731)	1273	138	88.29	+	1.56	5.62E-05
33.	mesoderm development (GO:0007498)	706	76	48.96	+	1.55	3.55E-02
34.	RNA metabolic process (GO:0016070)	2315	235	160.56	+	1.46	6.39E-07
35.	developmental process (GO:0032502)	2468	250	171.17	+	1.46	2.15E-07
36.	primary metabolic process (GO:0044238)	6997	651	485.28	+	1.34	1.16E-16
37.	metabolic process (GO:0008152)	8467	746	587.23	+	1.27	3.10E-14
38.	cellular process (GO:0009987)	7033	617	487.77	+	1.26	5.58E-10

39.	Unclassified (UNCLASSIFIED)	9561	500	663.1	-	0.75	0.00E+00
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Table S7 List of PANTHER GO-Slim biological processes revealed by PANTHER overrepresentation test associated with upregulated genes upon HP1 γ knockout in female MEFs. Over- or underrepresentation groups with $P < 0.05$ are presented. Definition of the PANTHER GO-Slim biological process can be found on the World Wide Web site for PANTHER classification system at: <http://www.pantherdb.org/>

	PANTHER GO-Slim Biological Process	No. of genes in mouse ref. list	No. of up- regulated genes in male	No. of expected genes	Over(+)/ under(-) repre- sentation	Fold enrich- ment	P-value
1.	regulation of liquid surface tension (GO:0050828)	55	10	2.08	+	4.81	1.37E-02
2.	cellular glucose homeostasis (GO:0001678)	62	10	2.34	+	4.27	3.59E-02
3.	cell-cell adhesion (GO:0016337)	362	48	13.69	+	3.51	4.85E-11
4.	ectoderm development (GO:0007398)	621	72	23.48	+	3.07	3.90E-14
5.	cell adhesion (GO:0007155)	550	60	20.8	+	2.88	1.76E-10
6.	homeostatic process (GO:0042592)	208	22	7.87	+	2.8	5.14E-03
7.	biological adhesion (GO:0022610)	577	60	21.82	+	2.75	1.21E-09
8.	nervous system development (GO:0007399)	782	81	29.57	+	2.74	2.28E-13
9.	muscle contraction (GO:0006936)	225	21	8.51	+	2.47	4.31E-02
10.	mesoderm development (GO:0007498)	706	59	26.7	+	2.21	5.52E-06
11.	system development (GO:0048731)	1273	106	48.14	+	2.2	1.11E-11
12.	cell-cell signaling (GO:0007267)	598	44	22.61	+	1.95	7.31E-03
13.	developmental process (GO:0032502)	2468	168	93.32	+	1.8	1.11E-11
14.	system process (GO:0003008)	1497	91	56.61	+	1.61	1.62E-03
15.	regulation of nucleobase- containing compound metabolic process (GO:0019219)	1621	98	61.3	+	1.6	8.77E-04
16.	single-	1828	107	69.12	+	1.55	1.24E-03

	multicellular organism process (GO:0044707)						
17.	regulation of transcription from RNA polymerase II promoter (GO:0006357)	1266	74	47.87	+	1.55	3.94E-02
18.	multicellular organismal process (GO:0032501)	1832	107	69.27	+	1.54	1.36E-03
19.	cell communication (GO:0007154)	3175	184	120.06	+	1.53	4.33E-07
20.	biological regulation (GO:0065007)	4101	207	155.07	+	1.33	1.14E-03
21.	cellular process (GO:0009987)	7033	345	265.94	+	1.3	1.35E-06
22.	Unclassified (UNCLASSIFIED)	9561	298	361.54	-	0.82	0.00E+00

Table S8 List of PANTHER GO-Slim biological processes revealed by PANTHER overrepresentation test associated with downregulated genes upon HP1 γ knockout in female MEFs. Over- or underrepresentation groups with $P < 0.05$ are presented. Definition of the PANTHER GO-Slim biological process can be found on the World Wide Web site for PANTHER classification system at: <http://www.pantherdb.org/>

	PANTHER GO-Slim Biological Process	No. of genes in mouse ref. list	No. of up-regulated genes in male	No. of expected genes	Over(+)/under(-) representation	Fold enrichment	P-value
1.	skeletal system development (GO:0001501)	241	11	1.72	+	6.41	3.43E-04
2.	visual perception (GO:0007601)	223	8	1.59	+	5.04	4.89E-02
3.	biological adhesion (GO:0022610)	577	14	4.11	+	3.41	1.65E-02
4.	cell adhesion (GO:0007155)	550	13	3.92	+	3.32	3.87E-02
5.	ectoderm development (GO:0007398)	621	14	4.42	+	3.16	3.54E-02
6.	mesoderm development (GO:0007498)	706	15	5.03	+	2.98	3.90E-02
7.	system development (GO:0048731)	1273	25	9.07	+	2.76	9.29E-04
8.	developmental process (GO:0032502)	2468	35	17.58	+	1.99	1.19E-02
9.	Unclassified (UNCLASSIFIED)	9561	55	68.11	-	0.81	0.00E+00

Table S9 List of the definition of the top 10 overrepresented PANTHER GO-Slim biological processes revealed by PANTHER overrepresentation test associated with upregulated genes upon HP1 γ knockout in male MEFs. Definition of the PANTHER GO-Slim biological process can be found on the World Wide Web site for PANTHER classification system at: <http://www.pantherdb.org/>

	Class ID	PANTHER GO-Slim Biological Process	Definition
1.	GO:0034341	response to interferon-gamma	"Any process that results in a change in state or activity of a cell or an organism (in terms of movement, secretion, enzyme production, gene expression, etc.) as a result of an interferon-gamma stimulus. Interferon-gamma is also known as type II interferon."
2.	GO:0019882	antigen processing and presentation	"The process in which an antigen-presenting cell expresses antigen (peptide or lipid) on its cell surface in association with an MHC protein complex."
3.	GO:0008283	cell proliferation	"The multiplication or reproduction of cells, resulting in the expansion of a cell population."
4.	GO:0019724	B cell mediated immunity	"Any process involved with the carrying out of an immune response by a B cell, through, for instance, the production of antibodies or cytokines, or antigen presentation to T cells."
5.	GO:0006968	cellular defense response	"A defense response that is mediated by cells."
6.	GO:0042116	macrophage activation	"A change in morphology and behaviour of a macrophage resulting from exposure to a cytokine, chemokine, cellular ligand, or soluble factor."
7.	GO:0006955	immune response	"Any immune system process that functions in the calibrated response of an organism to a potential internal or invasive threat."
8.	GO:0006644	phospholipid metabolic process	"The chemical reactions and pathways involving phospholipids, any lipid containing phosphoric acid as a mono- or diester."
9.	GO:0016337	cell-cell adhesion	"The attachment of one cell to another cell via adhesion molecules."
10.	GO:0005976	polysaccharide metabolic process	"The chemical reactions and pathways involving a polysaccharide, a polymer of many (typically more than 10) monosaccharide residues linked glycosidically."

Table S10 List of the definition of the top 10 overrepresented PANTHER GO-Slim biological processes revealed by PANTHER overrepresentation test associated with downregulated genes upon HP1 γ knockout in male MEFs. Definition of the PANTHER GO-Slim biological process can be found on the World Wide Web site for PANTHER classification system at: <http://www.pantherdb.org/>

	Class ID	PANTHER GO-Slim Biological Process	Definition
1.	GO:0051726	regulation of cell cycle	"Any process that modulates the rate or extent of progression through the cell cycle."
2.	GO:0006310	DNA recombination	"Any process in which a new genotype is formed by reassortment of genes resulting in gene combinations different from those that were present in the parents. In eukaryotes genetic recombination can occur by chromosome assortment, intrachromosomal recombination, or nonreciprocal interchromosomal recombination. Intrachromosomal recombination occurs by crossing over. In bacteria it may occur by genetic transformation, conjugation, transduction, or F-duction."
3.	GO:0006260	DNA replication	"The cellular metabolic process in which a cell duplicates one or more molecules of DNA. DNA replication begins when specific sequences, known as origins of replication, are recognised and bound by initiation proteins, and ends when the original DNA molecule has been completely duplicated and the copies topologically separated. The unit of replication usually corresponds to the genome of the cell, an organelle, or a virus. The template for replication can either be an existing DNA molecule or RNA."
4.	GO:0050828	regulation of liquid surface tension	"Any process that modulates the surface tension of a liquid. Surface tension is the property that makes a liquid behave as if it had an elastic skin on its surface at the interface with a gas or an immiscible liquid."
5.	GO:0007059	chromosome segregation	"The process in which genetic material, in the form of chromosomes, is organised into specific structures and then physically separated and apportioned to two or more sets. In eukaryotes, chromosome segregation begins with the alignment of chromosomes at the metaphase plate, includes chromosome separation, and

			ends when chromosomes have completed movement to the spindle poles."
6.	GO:0006281	DNA repair	"The process of restoring DNA after damage. Genomes are subject to damage by chemical and physical agents in the environment (e.g. UV and ionising radiations, chemical mutagens, fungal and bacterial toxins, etc.) and by free radicals or alkylating agents endogenously generated in metabolism. DNA is also damaged because of errors during its replication. A variety of different DNA repair pathways have been reported that include direct reversal, base excision repair, nucleotide excision repair, photoreactivation, bypass, double-strand break repair pathway, and mismatch repair pathway."
7.	GO:0006259	DNA metabolic process	"Any cellular metabolic process involving deoxyribonucleic acid. This is one of the two main types of nucleic acid, consisting of a long, unbranched macromolecule formed from one, or more commonly, two, strands of linked deoxyribonucleotides."
8.	GO:0006399	tRNA metabolic process	"The chemical reactions and pathways involving tRNA, transfer RNA, a class of relatively small RNA molecules responsible for mediating the insertion of amino acids into the sequence of nascent polypeptide chains during protein synthesis. Transfer RNA is characterized by the presence of many unusual minor bases, the function of which has not been completely established."
9.	GO:0000398	mRNA splicing, via spliceosome	"The joining together of exons from one or more primary transcripts of messenger RNA (mRNA) and the excision of intron sequences, via a spliceosomal mechanism, so that mRNA consisting only of the joined exons is produced."
10.	GO:0016072	rRNA metabolic process	"The chemical reactions and pathways involving rRNA, ribosomal RNA, a structural constituent of ribosomes."

Table 11 List of the definition of the top 10 overrepresented PANTHER GO-Slim biological processes revealed by PANTHER overrepresentation test associated with upregulated genes upon HP1 γ knockout in female MEFs. Definition of the PANTHER GO-Slim biological process can be found on the World Wide Web site for PANTHER classification system at: <http://www.pantherdb.org/>

	Class ID	PANTHER GO-Slim Biological Process	Definition
1.	GO:0050828	regulation of liquid surface tension	"Any process that modulates the surface tension of a liquid. Surface tension is the property that makes a liquid behave as if it had an elastic skin on its surface at the interface with a gas or an immiscible liquid."
2.	GO:0001678	cellular glucose homeostasis	"A cellular homeostatic process involved in the maintenance of an internal steady state of glucose within a cell or between a cell and its external environment."
3.	GO:0016337	cell-cell adhesion	"The attachment of one cell to another cell via adhesion molecules."
4.	GO:0007398	ectoderm development	"The process whose specific outcome is the progression of the ectoderm over time, from its formation to the mature structure. In animal embryos, the ectoderm is the outer germ layer of the embryo, formed during gastrulation."
5.	GO:0007155	cell adhesion	"The attachment of a cell, either to another cell or to an underlying substrate such as the extracellular matrix, via cell adhesion molecules."
6.	GO:0042592	homeostatic process	"Any biological process involved in the maintenance of an internal steady state."
7.	GO:0022610	biological adhesion	"The attachment of a cell or organism to a substrate or other organism."
8.	GO:0007399	nervous system development	"The process whose specific outcome is the progression of nervous tissue over time, from its formation to its mature state."
9.	GO:0006936	muscle contraction	"A process in which force is generated within muscle tissue, resulting in a change in muscle geometry. Force generation involves a chemo-mechanical energy conversion step that is carried out by the actin/myosin complex activity, which generates force through ATP hydrolysis."
10.	GO:0007498	mesoderm development	"The process whose specific outcome is the progression of the mesoderm over time, from its formation to the mature structure. The mesoderm is the middle germ layer that develops into muscle, bone, cartilage, blood and connective tissue."

Table S12 List of the definition of the overrepresented PANTHER GO-Slim biological processes revealed by PANTHER overrepresentation test associated with downregulated genes upon HP1 γ knockout in female MEFs. Definition of the PANTHER GO-Slim biological process can be found on the World Wide Web site for PANTHER classification system at: <http://www.pantherdb.org/>

	Class ID	PANTHER GO-Slim Biological Process	Definition
1.	GO:0001501	skeletal system development	"The process whose specific outcome is the progression of the skeleton over time, from its formation to the mature structure. The skeleton is the bony framework of the body in vertebrates (endoskeleton) or the hard outer envelope of insects (exoskeleton or dermoskeleton)."
2.	GO:0007601	visual perception	
3.	GO:0022610	biological adhesion	"The attachment of a cell or organism to a substrate or other organism."
4.	GO:0007155	cell adhesion	"The attachment of a cell, either to another cell or to an underlying substrate such as the extracellular matrix, via cell adhesion molecules."
5.	GO:0007398	ectoderm development	"The process whose specific outcome is the progression of the ectoderm over time, from its formation to the mature structure. In animal embryos, the ectoderm is the outer germ layer of the embryo, formed during gastrulation."
6.	GO:0007498	mesoderm development	"The process whose specific outcome is the progression of the mesoderm over time, from its formation to the mature structure. The mesoderm is the middle germ layer that develops into muscle, bone, cartilage, blood and connective tissue."
7.	GO:0048731	system development	"The process whose specific outcome is the progression of an organismal system over time, from its formation to the mature structure. A system is a regularly interacting or interdependent group of organs or tissues that work together to carry out a given biological process."
8.	GO:0032502	developmental process	"A biological process whose specific outcome is the progression of an integrated living unit: an anatomical structure (which may be a subcellular structure, cell, tissue, or organ), or organism over time from an initial condition to a later condition."
9.	N/A	Unclassified	N/A

Table S13 List of genes that contribute to the biological process ‘B cell mediated immunity’. Information obtained from the World Wide Web site for PANTHER classification system at: <http://www.pantherdb.org/>

	Gene symbol	Gene name
1.	Clec7a	C-type lectin domain family 7 member A
2.	Vav1	Proto-oncogene vav
3.	Slamf7	SLAM family member 7
4.	Cd22	B-cell receptor CD22
5.	Clec2h	C-type lectin domain family 2 member H
6.	Emr1	EGF-like module-containing mucin-like hormone receptor-like 1
7.	Klra3	Killer cell lectin-like receptor 3
8.	Gab3	GRB2-associated-binding protein 3
9.	Siglec1	Sialoadhesin;Siglec1
10.	Vav3	Guanine nucleotide exchange factor VAV3
11.	Slamf6	SLAM family member 6
12.	Il12rb2	Interleukin-12 receptor subunit beta-2
13.	Il13ra2	Interleukin-13 receptor subunit alpha-2
14.	Il12rb1	Interleukin-12 receptor subunit beta-1
15.	Gab2	GRB2-associated-binding protein 2
16.	Clec5a	C-type lectin domain family 5 member A
17.	Pecam1	Platelet endothelial cell adhesion molecule
18.	Cd33	Myeloid cell surface antigen CD33
19.	Tnfrsf17	Tumor necrosis factor receptor superfamily member 17
20.	Phldb3	Protein Phldb3
21.	Tnfrsf1b	Tumor necrosis factor receptor superfamily member 1B
22.	Ly9	T-lymphocyte surface antigen Ly-9
23.	Tnfrsf26	Tumor necrosis factor receptor superfamily member 26
24.	Polm	DNA-directed DNA/RNA polymerase mu
25.	Emr4	EGF-like module-containing mucin-like hormone receptor-like 4
26.	Clec12a	C-type lectin domain family 12 member A
27.	Csf3r	Granulocyte colony-stimulating factor receptor
28.	Plekhg1	Protein Plekhg1 (Fragment)
29.	Cd2	T-cell surface antigen CD2
30.	Cd84	SLAM family member 5
31.	Ngfr	Tumor necrosis factor receptor superfamily member 16
32.	Klra2	Killer cell lectin-like receptor 2
33.	Fcrl1	Fc receptor-like protein 1
34.	Ebi3	Interleukin-27 subunit beta
35.	Tnfrsf18	Tumor necrosis factor receptor superfamily member 18
36.	Sh2b2	SH2B adapter protein 2
37.	Arhgef6	Rho guanine nucleotide exchange factor 6
38.	Fcrlb	Fc receptor-like B
39.	Spata13	Spermatogenesis-associated protein 13

Table S14 List of genes that contribute to the biological process ‘Macrophage activation’. Information obtained from the World Wide Web site for PANTHER classification system at: <http://www.pantherdb.org/>

	Gene symbol	Gene name
1.	Mrc1	Macrophage mannose receptor 1
2.	Cd36	Platelet glycoprotein 4
3.	Pltp	Phospholipid transfer protein
4.	S100a13	Protein S100-A13
5.	Clec4a1	C-type lectin domain family 4, member a1
6.	Gbp7	Gbp6 protein
7.	Col20a1	Collagen alpha-1(XX) chain
8.	Msr1	Macrophage scavenger receptor types I and II
9.	Syk	Lysine--tRNA ligase
10.	Adra1a	Alpha-1A adrenergic receptor
11.	Lgals3bp	Galectin-3-binding protein
12.	Ccl28	C-C motif chemokine 28
13.	Gab3	GRB2-associated-binding protein 3
14.	Marco	Macrophage receptor MARCO
15.	Colec12	Collectin-12
16.	Col4a6	Protein Col4a6
17.	Gab2	GRB2-associated-binding protein 2
18.	Pecam1	Platelet endothelial cell adhesion molecule
19.	Bpifb6	BPI fold-containing family B member 6
20.	Bpifb4	BPI fold-containing family B member 4
21.	Gbp9	Guanylate-binding protein 9
22.	Phldb3	Protein Phldb3
23.	Cd5l	CD5 antigen-like
24.	Tnfrsf1b	Tumor necrosis factor receptor superfamily member 1B
25.	Tnfrsf26	Tumor necrosis factor receptor superfamily member 26
26.	Clec4e	C-type lectin domain family 4 member E
27.	Col4a3	Collagen alpha-3(IV) chain
28.	Cd209c	CD209 antigen-like protein C
29.	Syk	Tyrosine-protein kinase SYK
30.	Cxcl16	C-X-C motif chemokine 16
31.	S100a1	Protein S100-A1
32.	Ngfr	Tumor necrosis factor receptor superfamily member 16
33.	Adra2a	Alpha-2A adrenergic receptor
34.	Gbp4	Guanylate-binding protein 4
35.	Clec4a3	C-type lectin domain family 4, member a3
36.	Clec4d	C-type lectin domain family 4 member D
37.	Fcrl1	Fc receptor-like protein 1
38.	Tnfrsf18	Tumor necrosis factor receptor superfamily member 18
39.	Gbp8	Guanylate binding protein 8
40.	Adra2c	Alpha-2C adrenergic receptor
41.	Fcrlb	Fc receptor-like B
42.	Csf1	Macrophage colony-stimulating factor 1

Table S15 List of genes with higher expression in WT male compared to WT female MEFs (for at least 1.5 fold, P-value < 0.05)

	Genes	Chromosome
1.	<i>Eif2s3y</i>	Y
2.	<i>Ddx3y</i>	Y
3.	<i>Kdm5d</i>	Y
4.	<i>Uty</i>	Y
5.	<i>Erdr1</i>	Y
6.	<i>Gpr165</i>	X
7.	<i>Lrrc10b</i>	19
8.	<i>Dkk1</i>	19
9.	<i>Tslp</i>	18
10.	<i>Pi16</i>	17
11.	<i>Chodl</i>	16
12.	<i>Col2a1</i>	15
13.	<i>Krt75</i>	15
14.	<i>Fgf9</i>	14
15.	<i>Gjb6</i>	14
16.	<i>Thbs4</i>	13
17.	<i>Hapln1</i>	13
18.	<i>Adcy2</i>	13
19.	<i>Dlk1</i>	12
20.	<i>Syndig1l</i>	12
21.	<i>Klhl29</i>	12
22.	<i>2810032G03Rik</i>	12
23.	<i>Ramp2</i>	11
24.	<i>Ace</i>	11
25.	<i>Krt13</i>	11
26.	<i>Krt14</i>	11
27.	<i>S100b</i>	10
28.	<i>Susd2</i>	10
29.	<i>Bmp5</i>	9
30.	<i>Stac</i>	9
31.	<i>1700048O20Rik</i>	9
32.	<i>Gsta4</i>	9
33.	<i>Gramd2</i>	9
34.	<i>1700102P08Rik</i>	9
35.	<i>Cdh8</i>	8
36.	<i>Clgn</i>	8
37.	<i>Lpl</i>	8
38.	<i>Acan</i>	7
39.	<i>Scube2</i>	7
40.	<i>Hif3a</i>	7
41.	<i>Ntrk3</i>	7
42.	<i>Gpr27</i>	6
43.	<i>Klf14</i>	6

44.	<i>Tril</i>	6
45.	<i>Eln</i>	5
46.	<i>Col8a2</i>	4
47.	<i>Frem1</i>	4
48.	<i>Col9a2</i>	4
49.	<i>Ptx3</i>	3
50.	<i>Agtr1b</i>	3
51.	<i>Sfrp2</i>	3
52.	<i>Snord73a</i>	3
53.	<i>Fam83c</i>	2
54.	<i>Mylk2</i>	2
55.	<i>Pcsk2</i>	2
56.	<i>Adamtsl2</i>	2
57.	<i>Wnt6</i>	1
58.	<i>Pappa2</i>	1
59.	<i>Col9a1</i>	1
60.	<i>Gsta3</i>	1
61.	<i>Angptl1</i>	1
62.	<i>Darc</i>	1

Table S16 List of genes with higher expression in WT female compared to WT male MEFs (for at least 1.5 fold, P-value < 0.05)

	GeneID	Chromosome
1.	Xist	X
2.	Eif2s3x	X
3.	5530601H04Rik	X
4.	Ppef1	X
5.	Gda	19
6.	Malat1	19
7.	Neat1	19
8.	4933411K16Rik	19
9.	Pcdha10	18
10.	9430076G02Rik	18
11.	Mc2r	18
12.	Gm4013	18
13.	Pcdha9	18
14.	Abcg1	17
15.	Rgs11	17
16.	Xdh	17
17.	Pisd-ps2	17
18.	C2	17
19.	Rhbdl1	17
20.	Stap2	17
21.	H2-DMb1	17
22.	1700071M16Rik	17

23.	Liph	16
24.	4930565N06Rik	16
25.	Nlrc3	16
26.	Kcnj15	16
27.	Kng2	16
28.	Slc7a4	16
29.	Kifc2	15
30.	Parp10	15
31.	Cma2	14
32.	Ptger2	14
33.	Gzmd	14
34.	Gzmc	14
35.	Mcpt8	14
36.	Hist1h3d	13
37.	Fam228b	12
38.	4930447C04Rik	12
39.	F730043M19Rik	12
40.	Ccl8	11
41.	Doc2b	11
42.	Pisd-ps1	11
43.	Rasl10b	11
44.	Fbxw10	11
45.	Sfi1	11
46.	Bzrap1	11
47.	Cntnap1	11
48.	Havcr2	11
49.	Slc16a11	11
50.	Arg1	10
51.	Hal	10
52.	Gstt2	10
53.	Gm5134	10
54.	Acp5	9
55.	Fxyd2	9
56.	Cxcr6	9
57.	Mmp3	9
58.	Cck	9
59.	Cmtm8	9
60.	Pstpip1	9
61.	Mmp13	9
62.	Mmp10	9
63.	Hapln4	8
64.	Chst5	8
65.	Mboat4	8
66.	Insl3	8
67.	Snord68	8
68.	Acta1	8
69.	Lrp2bp	8
70.	Slc27a1	8

71.	Slc5a5	8
72.	Nlrc5	8
73.	Gpr123	7
74.	Syt17	7
75.	2810047C21Rik1	7
76.	Apoc2	7
77.	Tnfrsf26	7
78.	Crym	7
79.	0610005C13Rik	7
80.	Calcb	7
81.	Fam19a1	6
82.	Clec2e	6
83.	Vwf	6
84.	B230378P21Rik	6
85.	Alox5	6
86.	Spp1	5
87.	Plac8	5
88.	Fbxo24	5
89.	Ppbp	5
90.	Tfr2	5
91.	Bmp8b	4
92.	Cdkn2a	4
93.	Rex2	4
94.	Tas1r1	4
95.	Angptl7	4
96.	1300002K09Rik	4
97.	Tnfrsf18	4
98.	Rnf207	4
99.	Car9	4
100.	LOC100038947	3
101.	Tgm2	2
102.	Angpt4	2
103.	Slc52a3	2
104.	Gm996	2
105.	1700020A23Rik	2
106.	Rhov	2
107.	Siglec1	2
108.	Olf1r1316	2
109.	Htr2b	1
110.	Otos	1
111.	Nphs2	1
112.	Kcnh1	1
113.	Fam5c	1
114.	Creg2	1

Table S17 List of upregulated (for at least 1.5 fold, adjusted P-value < 0.05) imprinted genes upon HP1 γ knockout in male MEFs.

	Loci Symbol	Loci Name	Chromosome	Expressed Parental Allele	Fold Change (UP)	Adjusted P-value
1.	<i>Ampd3</i>	adenosine monophosphate deaminase 3	7	M	3.92602	3.78E-11
2.	<i>Ascl2</i>	achaete-scute complex homolog 2 (Drosophila)	7	M	2.57948	0.034654
3.	<i>Calcr</i>	calcitonin receptor	6	M	6.02507	0.013862
4.	<i>Cntn3</i>	contactin 3	6	M	2.70799	0.012574
5.	<i>Cobl</i>	cordon-bleu WH2 repeat	11	M	1.57141	0.018566
6.	<i>Gatm</i>	glycine amidinotransferase (L-arginine: glycine amidinotransferase)	2	M	2.10672	0.000467
7.	<i>Pon2</i>	paraoxonase 2	6	M	2.53656	2.59E-20
8.	<i>Pon3</i>	paraoxonase 3	6	M	2.48782	3.1E-06
9.	<i>Ppp1r9a</i>	protein phosphatase 1, regulatory (inhibitor) subunit 9A	6	M	5.07041	3.05E-16
10.	<i>Qpct</i>	glutaminyl-peptide cyclotransferase (glutaminyl cyclase)	17	M	1.72828	2.18E-09
11.	<i>Scin</i>	scinderin	12	M	11.6446	1.48E-05
12.	<i>Slc22a18</i>	solute carrier family 22 (organic cation transporter), member 18	7	M	2.23488	4.87E-05
13.	<i>Tnfrsf23</i>	tumor necrosis factor receptor superfamily, member 23	7	M	1.88413	6.26E-06
14.	<i>Trappc9</i>	trafficking protein particle complex 9	15	M	1.50205	4.93E-12
15.	<i>Xlr4b</i>	X-linked lymphocyte-regulated 4B	X	M	3.11448	0.00039
16.	<i>Xlr4c</i>	X-linked lymphocyte-regulated 4C	X	M	3.52326	0.020336
17.	<i>Rasgrf1</i>	RAS protein-specific guanine nucleotide-releasing factor 1	9	P	6.24269	1.23E-05

Table S18 List of downregulated (for at least 1.5 fold, adjusted P-value < 0.05) imprinted genes upon HP1 γ knockout in male MEFs.

	Loci Symbol	Loci Name	Chromosome	Expressed Parental Allele	Fold Change (DOWN)	Adjusted P-value
1.	<i>AF357426</i>	AF357426		M	2.29706	0.041699
2.	<i>Asb4</i>	ankyrin repeat and SOCS box-containing 4	6	M	1.7423	0.038
3.	<i>Atp10a</i>	ATPase, class V, type 10A	7	M	4.3064	3.35E-18
4.	<i>Cdkn1c</i>	cyclin-dependent kinase inhibitor 1C (P57)	7	M	6.24367	2.04E-07
5.	<i>Dcn</i>	decorin	10	M	2.36107	0.030233
6.	<i>Dhcr7</i>	7-dehydrocholesterol reductase	7	M	1.74554	0.000205
7.	<i>Dlx5</i>	distal-less homeobox 5	6	M	1.80285	0.013625
8.	<i>Grb10</i>	growth factor receptor bound protein 10	11	M	3.35366	1.08E-34
9.	<i>H19</i>	H19, imprinted maternally expressed transcript	7	M	13.3301	8.59E-67
10	<i>Htr2a</i>	5-hydroxytryptamine (serotonin) receptor 2A	14	M	1.69666	0.000151
11	<i>Igf2r</i>	insulin-like growth factor 2 receptor	17	M	2.15607	2.81E-16
12	<i>Klf14</i>	Kruppel-like factor 14	6	M	12.8701	4.23E-05
13	<i>Meg3</i>	maternally expressed 3	12	M	1.53386	0.033964
14	<i>Rian</i>	RNA imprinted and accumulated in nucleus	12	M	2.34174	1.29E-10
15	<i>Tfpi2</i>	tissue factor pathway inhibitor 2	6	M	2.5143	0.012203
16	<i>Zim1</i>	zinc finger, imprinted 1	7	M	3.71557	3.36E-08
17	<i>Air</i>	antisense Igf2r RNA	17	P	1.96305	5.86E-05
18	<i>Begain</i>	brain-enriched guanylate kinase-associated	12	P	2.92865	2.39E-06
19	<i>Dio3</i>	deiodinase, iodothyronine type III	12	P	4.16246	2.85E-07
20	<i>Dlk1</i>	delta-like 1 homolog (Drosophila)	12	P	12.7392	2.84E-10
21	<i>Igf2</i>	insulin-like growth factor 2	7	P	12.9434	4.78E-63
22	<i>Magel2</i>	melanoma	7	P	2.83145	0.015885

		antigen, family L, 2				
23	<i>Nap1l5</i>	nucleosome assembly protein 1-like 5	6	P	1.98772	0.006838
24	<i>Nnat</i>	neuronatin	2	P	1.67783	0.012808
25	<i>Peg12</i>	paternally expressed 12	7	P	1.60317	3.07E-05
26	<i>Peg3</i>	paternally expressed 3	7	P	3.14558	7.9E-08
27	<i>Plagl1</i>	pleiomorphic adenoma gene-like 1	10	P	3.66751	1.31E-10
28	<i>Rtl1</i>	retrotransposon-like 1	12	P	2.9626	0.006971
29	<i>Sfmbt2</i>	Scm-like with four mbt domains 2	2	P	1.61025	5.49E-05
30	<i>Slc38a4</i>	solute carrier family 38, member 4	15	P	9.74479	1.61E-48
31	<i>Usp29</i>	ubiquitin specific peptidase 29	7	P	2.38313	0.006319
32	<i>Zdbf2</i>	zinc finger, DBF-type containing 2	1	P	7.29949	2.46E-10

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