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The epigenetic regulation of heterochromatin structure and tumour progression

Peter Christopher Bruton

Thesis presented for the degree of Doctor of Philosophy

The University of Edinburgh

2018
Declaration

I declare that this thesis is of original work almost entirely undertaken by the author. Where collaborative contributions are included, they have been explicitly stated and acknowledged. All external sources have been specifically referenced where required. This body of work has not been submitted in any part to support any other degree or qualification.

Peter Bruton

March 2018
Acknowledgements

Ask any doctorate to describe what it’s like to do a PhD, and you are inevitably informed that it is only comprehensible by those who have been there and done it. The unerring accuracy of ‘PHDComics’ is often quoted as a reference to those naïve enough to consider it. And yet here we are.

Researching and writing this thesis has frankly often felt like a car crash. From a so-called rabbit in the headlights, to the insomniac that may finally venture outside again, it has been a transformative process. That I live to tell the tale speaks only to the limits that ‘doing a PhD’ may take from someone. Despite my future calling lying away from academia, taking on this thesis – my Everest – will never be a regret.

“People are like dirt. They can either nourish you and help you grow as a person or they can stunt your growth and make you wilt and die.”

- Anon (falsely attributed to Plato)

This body of work has required an endless supply of support, from too many sources to mention in full. Firstly, I would like to thank the MRC for funding my research, and Professor Nick Gilbert for taking on my project when I was already figuratively in intensive care. The scientific rigour and discussions within his lab dragged my project off the tarmac and to the finish line today.

I would also like to thank my supporting supervisors in Neil Carragher and Alexander ‘Sasha’ Kagansky. Neil’s insightful commentary and advice on the broader implications of my research, helped frame my thoughts. I would also like to thank Sasha for his stupendous positivity and enthusiasm, which reminded me that exploring life’s unanswered questions should remain a fun and inspiring process.
Covadonga Huidobro-Fernandez provided much needed technical and emotional support to which I am hugely indebted. Her passion, smile, Polaroid camera, and endless organisational abilities kept me on the straight and narrow. Without her I would still be rummaging around the back of the wrong freezer looking for ‘that reagent’ I had managed to misplace.

The exceptional colleagues within the Gilbert lab created a supportive environment of great benefit to this project and myself. From the perfect role model in Ryu-ske to Jim’s notable wisdom; Lora, Adam, Catherine, Sam, Hannah, Kate, and Maria have all generously supported this work and put up with my questionable music tastes in the lab.

I would be remised not to mention all the support provided by those outside the lab, from the Genome Regulation section and the wider IGMM. I have greatly benefitted from Matt Pearsons advice and training in microscopy, Lizzy Freyer for FACS support, Bernie Ramsahoye for mononucleotide analyses, and Technical Services for freedom from resource acquisition and stock solution preparations.

The greatest thanks, however, goes to my friends and family for feigning interest in my work, putting up with my enthusiastic and informing scientific monologues, and generally avoiding the question ‘How’s the PhD going?’ I would finally like to say thank you to the unwavering constant that is my mum and dad. The lifetime of scientific discussions/arguments, mountainous amounts of proofreading, and unconditional support have made me the man I am today.
Lay Summary

DNA is packaged into ‘on’ and ‘off’ states, which are loosely more open and accessible and more compact and inaccessible respectively. How the compact heterochromatic ‘off’ state is regulated and its role in disease is poorly understood.

The ‘off’ heterochromatic state has defined characteristics of increased DNA methylation and Histone 3 trimethylation (H3K9me3). DNA methylation loss does not change the compact nature of heterochromatin. Loss of H3K9me3 results in an increase in cancer in mice; however visually compact DNA is still present.

Broadly I aimed to identify components that regulate the heterochromatic ‘off’ state, and investigate their role in the development of colon cancer drug resistance. I found that the loss of a component that turns ‘on’ genes appeared to regulate silent genes. This corresponded to a decrease in HP1 that is normally associated with maintaining the heterochromatic ‘off’ associated. This suggests that the activator is required to produce the components to maintain the compact ‘off’ state.

Additionally I aimed to address whether H3K9me3 effects the compaction of the heterochromatic ‘off’ state. I found that loss of H3K9me3 affected the level of compaction globally as well as at compact ‘off’ regions of the DNA. This correlated with an increase the amount of RNA bound to DNA. Re-establishing H3K9me3 ablated these effects. Further research is required to establish how H3K9me3 affects DNA bound RNA.
Abstract

Since the discovery of DNA packaging into chromatin, and McClintock’s (1951) work on position-effect variegation providing evidence of non-mendelian inheritance, the principal of a genome maintaining ‘on’ and ‘off’ states has been widely adopted. However, the underlying mechanisms that regulate these dynamic chromatin states and their effect on disease are still poorly understood.

DNA methylation and histone trimethylation at H3K9 and H4K20 are the core hallmarks of the heterochromatic constitutively ‘off’ state. Constitutive heterochromatin is predominantly comprised of repetitive satellite containing pericentromeric regions and telomeres and in mouse heterochromatin clusters into large chromocenters. These regions are cytologically more compact and generally transcriptionally silent across embryonic and differentiated mouse cell types. However, in addition to increased genomic instability, mouse tumour cells sustain increased satellite expression suggesting constitutive heterochromatin is disrupted. Therefore how constitutive heterochromatin is maintained has important implications for genome regulation and disease, and remains poorly understood.

While satellite DNA sequences are not evolutionarily conserved, pericentromeric and telomeric heterochromatin occurs across species. Heterochromatin formation is therefore independent of the underlying DNA sequence, supporting the hypothesis that epigenetic components can regulate chromatin structure. DNA methylation is generally thought to be associated with transcriptional silencing and chromatin compaction.
However, Gilbert et al. (2007) showed that the complete loss of DNA methylation did not affect the compaction at heterochromatin or global genome compaction.

The role of H3K9me3 in regulating heterochromatin has also been an area of keen interest. H3K9me3 patterns are established by suppressor of variegation 3-9 homologues and provide the binding site for heterochromatic protein 1 [HP1] which can in turn recruit Suv39h1. This Suv3-9h-HP1-H3K9 axis enables its propagation throughout heterochromatin. Peters et al. (2001) demonstrated that in mice loss of suv39 homologues 1 and 2 caused a loss of H3K9me3 at constitutive heterochromatic domains. These Suv39h null mice demonstrated decreased genome stability, and an increased prevalence of oncogenesis. However cytological chromocenters are still present in the absence of H3K9me3. Therefore the function of H3K9me3 as a causative agent in heterochromatin formation is still debated.

Broadly the aim was to investigate the phenotypic role of heterochromatic epigenetic components in cancer progression, and address whether H3K9me3 effects large scale chromatin structure. To identify heterochromatic gene silencing components, an inhibitor screen was performed in an artificial silenced reporter system. The reporter fluorophore was silenced by the presence of centromeric arrays from yeast/bacterial artificial chromosomes and human alpha satellite repeats enriched for H3K9me3. To address the function of the de-silencing components identified in cancer, the fitness of colon cancer cells [HCT116] was investigated before and after the development of resistance to the MEK inhibitor trametinib. The most intriguing result was that BET protein inhibition resulted in de-repression of the reporter construct and trametinib resistant HCT116 cells.
were more sensitive to BET inhibitors, while subsequent investigation showed HP1 protein levels were altered. Analysis of publically available datasets of tumour drug resistance, showed elevated BET protein binding at HP1 promoters in resistant cell lines suggesting an indirect role in gene silencing.

To investigate the consequence of H3K9me3 loss on chromatin structure, mouse embryonic stem cells that lacked both Suv39 homologues were used. Microccocal nuclease digestion and sucrose sedimentation demonstrated a global decompaction of large-scale chromatin fibres whilst re-expression of suv39h1 rescued H3K9me3 at chromocenters and global chromatin decompaction. Loss of Suv39h also increased chromatin associated RNA levels that were also rescued by Suv39h1 re-expression. This suggests that H3K9me3 has a role chromatin fibre compaction globally as well as at constitutive heterochromatin, potentially mediated by chromatin associated RNA.

To conclude, multiple components were identified that are involved in transcriptional silencing. Evaluating their function in tumour progression demonstrated a possible role of BET proteins in the development of MEKi resistance that may be mediated through HP1 proteins. H3K9me3 and its binding partner HP1 affect global chromatin compaction. The global decompaction after Suv39h loss correlates with an increase in chromatin associated RNA, suggesting a possible mechanism for changes in chromatin compaction beyond H3K9me3.
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Abbreviations

3C: one vs one chromosome conformation capture
BET: Bromo extra-terminal domain
bp: base pairs
BrdU: 5-bromo-2-deoxy-uridine
BSA: bovine serum albumin
cDNA: complementary DNA
CdLS: cornelia de lange syndrome
CHD: chromo helicase domain
ChIP: chromatin immunoprecipitation
Coumarin-azide: 3-azido-7-hydroxycoumarin
DAPI: 4’,6-diamino-2-phenylindole
DMSO: dimethyl sulfoxide
DNA deoxyribonucleic acid
DnaseI: deoxyribonucleic acid nuclease 1
dH₂O: distilled water
dNTP: deoxyribonucleotide triphosphate
DTT: dithiothreitol
eGFP: enhanced green fluorescence protein
EDTA: ethylene-diamine-tetraacetic acid
EdC: 5-ethynyl-2’deoxy-cytidine
EdU: 5-ethynyl-2’deoxy-uridine
EGTA: ethylene glycol tetraacetic acid
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tr>
<td>EMT</td>
<td>epithelial mesenchymal transition</td>
</tr>
<tr>
<td>ENC</td>
<td>evolutionary new centromere</td>
</tr>
<tr>
<td>eRNA</td>
<td>enhancer RNA</td>
</tr>
<tr>
<td>EU</td>
<td>5-ethynyl-2’uridine</td>
</tr>
<tr>
<td>FA</td>
<td>formaldehyde</td>
</tr>
<tr>
<td>FACS</td>
<td>fluorescence-activated cell sorting</td>
</tr>
<tr>
<td>FCS</td>
<td>fetal calf serum</td>
</tr>
<tr>
<td>FISH</td>
<td>fluorescence in situ hybridization</td>
</tr>
<tr>
<td>H1</td>
<td>linker histone 1</td>
</tr>
<tr>
<td>HAC</td>
<td>human artificial chromosome</td>
</tr>
<tr>
<td>HATs</td>
<td>histone acetyltransferases</td>
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<tr>
<td>HDACs</td>
<td>histone deacetylases</td>
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<td>Hi-C</td>
<td>all vs all chromosome conformation capture</td>
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<td>HMTs</td>
<td>histone methyltransferases</td>
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<td>HKMTs</td>
<td>Lysine methyltransferases</td>
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<tr>
<td>HJQ1R</td>
<td>JQ1 resistant HCT116 cells</td>
</tr>
<tr>
<td>hnRNPs</td>
<td>heterogeneous nuclear ribonucleoproteins</td>
</tr>
<tr>
<td>HP1</td>
<td>heterochromatic protein 1</td>
</tr>
<tr>
<td>HPLC</td>
<td>high performance liquid chromatography</td>
</tr>
<tr>
<td>HR</td>
<td>homologous repair</td>
</tr>
<tr>
<td>HRP</td>
<td>horseradish peroxidase</td>
</tr>
<tr>
<td>HSA</td>
<td>highest single agent model</td>
</tr>
<tr>
<td>IC50</td>
<td>inhibitory concentration 50</td>
</tr>
<tr>
<td>IRES</td>
<td>internal ribosome entry site</td>
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<td>Description</td>
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<td>--------------</td>
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</tr>
<tr>
<td>kb</td>
<td>kilobase</td>
</tr>
<tr>
<td>LAD</td>
<td>lamina associated domain</td>
</tr>
<tr>
<td>LCR</td>
<td>locus control region</td>
</tr>
<tr>
<td>IncRNA</td>
<td>long non-coding RNA</td>
</tr>
<tr>
<td>M</td>
<td>molar</td>
</tr>
<tr>
<td>me1/me2/me3</td>
<td>monomethylation/dimethylation/trimethylation</td>
</tr>
<tr>
<td>MEF</td>
<td>mouse embryonic fibroblasts</td>
</tr>
<tr>
<td>MEKi</td>
<td>MEK inhibitors</td>
</tr>
<tr>
<td>MEL</td>
<td>murine erythroleukaemia</td>
</tr>
<tr>
<td>mESCs</td>
<td>mouse embryonic stem cells</td>
</tr>
<tr>
<td>MET</td>
<td>mesenchymal endothelial transition</td>
</tr>
<tr>
<td>ml</td>
<td>millilitre</td>
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<td>milliMolar</td>
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<td>MNase</td>
<td>micrococcal nuclease</td>
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<td>non-coding RNA</td>
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<td>non-homologous end joining</td>
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<td>nanoMolar</td>
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<tr>
<td>nm</td>
<td>nanometer</td>
</tr>
<tr>
<td>O.N</td>
<td>overnight</td>
</tr>
<tr>
<td>p212</td>
<td>MEL cells with constitutively active eGFP transgene</td>
</tr>
<tr>
<td>PARP</td>
<td>poly(ADP-ribose) polymerase</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffer saline</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
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<td>Abbreviation</td>
<td>Definition</td>
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<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>PEV</td>
<td>Position-effect variegation</td>
</tr>
<tr>
<td>PFA</td>
<td>paraformaldehyde</td>
</tr>
<tr>
<td>PcG</td>
<td>polycomb complexes</td>
</tr>
<tr>
<td>PI</td>
<td>propidium iodide</td>
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<td>PIC</td>
<td>preinitiation complex</td>
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<td>RNA polymerase II</td>
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<tr>
<td>PRC2</td>
<td>polycomb repressor complex 2</td>
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<td>PMSF</td>
<td>phenylmethylsulfonyl fluoride</td>
</tr>
<tr>
<td>PTM</td>
<td>post-translational modification</td>
</tr>
<tr>
<td>PVDF</td>
<td>polyvinylidene fluoride</td>
</tr>
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<td>RFI</td>
<td>relative fluorescence intensity</td>
</tr>
<tr>
<td>RFP</td>
<td>red fluorescent protein</td>
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<td>receptor tyrosine kinases</td>
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<td>RL5</td>
<td>endogenous MEL cells</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>RNase</td>
<td>ribonucleic nuclease</td>
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<tr>
<td>ROI</td>
<td>region of interest</td>
</tr>
<tr>
<td>rpm</td>
<td>revolutions per minute</td>
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<tr>
<td>RT</td>
<td>room temperature</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulphate</td>
</tr>
<tr>
<td>SET</td>
<td>su(var)3-9, enhancer-of-zeste and trithorax</td>
</tr>
<tr>
<td>SEM</td>
<td>standard error of the mean</td>
</tr>
<tr>
<td>SMC</td>
<td>structural maintenance of chromosome</td>
</tr>
</tbody>
</table>
Su(var): suppressor of variegation
Suv39h1/2: suppressor of variegation 3-9 homologue 1 or 2
SUMOs: small ubiquitin-like modifiers
TADs: topological associating domains
telRNA: telomeric RNA
TF: transcription factor
THPTA: tris(3-hydroxypropyltriazolylmethyl)amine
TJQ1R: JQ1 and trametinib resistant HCT116 cells
Top1: type 1 topoisomerases
Top2: type 2 topoisomerases
TRAMR: trametinib resistant
Tris: tris(hydroxymethyl)aminomethane
TSA: trichostatin A
UV: ultraviolet
v/v: volume:volume
w/v: weight:volume
YAC: S. cerevisae artificial chromosomes
α-MEL: MEL cells with alpha satellite eGFP transgene
μM: micromolar
Chapter 1: Introduction

Since Friedrich Miescher discovered DNA in 1869 (Hoppe-Seyler, 1871) the structure and regulation of a cell’s genetic material has remained a fundamental research question. For Watson and Crick’s DNA structure to fit inside the nucleus of a eukaryotic cell the DNA requires multiple levels of packaging. DNA is initially wrapped around and bound to positively charged proteins forming fibres, which undergo further levels of folding.

The tight packaging of these fibres enables regulation of underlying DNA processes in addition to overcoming the spatial constraints of the nucleosome. This regulatory ability of fibre structure has been linked to gene expression, genome regulation, and repair pathways. Mechanisms to modulate the topology of chromatin fibres are loosely termed epigenetic, derived from the Greek term epi- meaning “on top of” genetics. These processes enable cells to differentiate into different sub types despite the same underlying genetic sequence.

The structure and regulation of condensed chromatin fibres is implicated in many biological processes, from faithful sister chromatid separation after replication to preventing gene expression at specific loci or in large Mb domains. For oncogenesis to occur tumour suppressor mechanisms must be disrupted, allowing uncontrolled oncogenic proliferation. DNA mutations, gene silencing, and gene activation mediate this process. Epigenetic pathways were first implicated in cancer when tumours were found to exhibit DNA hypomethylation (Feinberg and Vogelstein, 1983). With the addition of whole-genome sequencing, epigenetic components have been
demonstrated as the most frequently mutated in cancer (Forbes et al., 2011). Metastatic tumour progression after initial treatment is often resistant to treatment and lacks common new mutations like initial oncogenesis. This has led to the proposal of epigenetic mechanisms in metastasis formation.

Studying epigenetic components that regulate a condensed fibre state provides insight into fundamental genome organisation in addition to its phenotypic relevance.
1.1 Chromatin constituents

Multiple levels of organisation can define the complexity of chromatin structure. Primarily 147 base pairs of the DNA helix are wrapped 1.7 times around an octamer of histone proteins forming a core particle. In chromatin this structure is stabilised by histone H1 that binds close to the dyad, the point at which DNA enters and exits the nucleosome. Nucleosomes are positioned regularly along the DNA and when unfolded appear as a 10-nm ‘beads on a string’ like structure (Alberts, Bray and Lewis, 1994). Under physiological conditions nucleosome arrays are further packaged into 30-nm diameter fibres. Additional levels of folding have been argued in the order of 40-60 nm and 117 nm (Pope et al., 2005). Ultimately chromatin fibres are also folded by a looping model (Gibcus et al., 2018), which during mitosis results in the chromosome cytological bodies first seen by Walter Flemming (1878).

1.1.1 Core histones

The base unit of chromatin, the nucleosome comprises eight core histones and a linker histone. Linker histones bind to the DNA and associate with neighbouring nucleosomes through the long c-terminal tail (Allan et al., 1986). Core and linker histones, have many post-translational modifications [PTMs], including acetylation, methylation, phosphorylation, ubiquitination, and less well studied more “exotic” modifications. These modifications correlate with transcriptional states and it has been suggested they could influence chromatin fibre function. Along with PTMs, core histones of the nucleosome can be evicted and replaced with variants to regulate chromatin structure in a site-specific manner.
The histone octamer that forms a nucleosome when bound to DNA comprises of histones H2A, H2B, H3, H4, or their individual variants. H3 and H4 form a heterotetramer that binds to two H2A/H2B dimers. The histone octamer proteins are enriched with lysine and arginine residues resulting in a net positive charge whilst the antipodal negative charge of the DNA phosphate backbone results in a general propensity for DNA histone interactions.

DNA and histones interactions are confined by proximal electrostatic and Van de Waal forces on the surface of the histone globular domain, which results in the DNA wrapping around the histone octamer. These interaction forces also differ depending on the underlying nucleic acid sequence revealing a bias for nucleosome positioning (Gonzalez et al., 2016).

Neighbouring nucleosomes are individually distinct due to linker DNA being enriched with GC, GG, CG, and CC dinucleotide sequences. The DNA length of these linker regions fluctuates from 10-90 bp throughout the genome, by cell type, and species (Van Holdebo, 1989). Linker histones H1 and its variant H5 also contain polar and charged surface amino acid residues, which allow binding to linker DNA. The linker histone H1 is composed of a globular domain with C-terminal and N-terminal unstructured tails. The globular domain of H1 associates with the dyad of the nucleosome, while the disordered tails interact with the naked linker DNA and nearby nucleosomes. H1 binding stabilises nucleosomes by reducing the free energy of the supramolecule. This increases the tolerance for nucleosome stacking and compaction to a 30 nm fibre state (Allan et al., 1980).
1.1.2 Histone variants

Canonical histone genes cluster in the genome and their transcription activity is tightly regulated by the cell cycle. Histone variants, by contrast, are typically dispersed throughout the genome, and are constitutively expressed. Histone variant chromatin incorporation occurs at the expense of their canonical counterparts and is normally regulated by histone chaperones. Chaperones associated with additional complexes with preferential binding affinities to particular post-translational modifications, allows for histone variant deposition to be tightly regulated (Luk et al., 2007; Obri et al., 2014). Histone variants maintain a large percentage of sequence similarity; yet have multiple effects including altering the structural dynamics of chromatin (Talbert and Henikoff, 2017). This enables variants to have diverse functions in DNA repair, transcriptional, and replication processes.

H2A, H2B, H3 and H1 have known histone variants. Centromeric CENP-A, one of eight H3 variants, has greater than 60% similarity but unique N-terminal tails. Deposited by histone chaperone HJURP, CENP-A incorporated nucleosomes in vitro are more stable and form more compact fibres than canonical H3 (Panchenko et al., 2011). In vivo, however, CENP-A nucleosomes are interspersed, except at structurally similar inactive centromeres. CENP-A is thought, therefore, to stabilise the fibre structure allowing for correct kinetochore formation (Blower, Sullivan and Karpen, 2002).
Other H3 variants of note include structurally similar H3.1/2 and H.3.3. Differing via a single amino acid H3.1 and H3.2 have yet to have distinguished functions, and are together described as canonical H3. Canonical H3 incorporation is mediated by CAF-1 in a replication dependent manner, representing the majority of H3 in chromatin. H3.3 DNA sequence diverges from H3.1 by five base pairs in humans, resulting in less stable nucleosomes, suggesting an altered chromatin incorporation and effect on chromatin structure (Tagami et al., 2004; Chen et al., 2013).

H3.3 is deposited independently of the cell cycle by chaperone HIRA, or the chromatin re-modellers CHD1, and DAXX (Konev et al., 2007; Drané et al., 2010). H3.3 is enriched at enhancer and transcribed loci, suggesting it might be frequently turned over, although it is also enriched at telomeric and pericentromeric regions (Wirbelauer and Bell, 2005). H3.3 enrichment at active and inactive regions is dependent on HIRA or DAXX mediated incorporation respectively (Goldberg et al., 2010).

H2A variants have a diverse range of functions. H2A.Z is deposited at actively transcribed genes and is often found at the same loci as H3.3. Incorporation of H2A.Z destabilises nucleosomes synergistically with H3.3 resulting in nucleosome depleted regions thought to promote transcription factor binding (Suto et al., 2000; Jin et al., 2011; Chen et al., 2013). The local chromatin environment also regulates the DNA damage response, through the replacement of H2A by H2A.X. Distributed throughout the genome, this variant has an alternate C-terminal, which upon DNA damage is phosphorylated at ser139 marking the loci for repair (Rogakou et al., 1998). The most divergent variant is macro-H2A, which is nearly three times larger than canonical H2A.
Macro-H2A has 64% similarity to H2A, but also contains a linker to a large ancillary C-terminal domain that is located outside the core nucleosome particle (Pehrson and Fried, 1992). Found at the inactive X chromosome, macro-H2A is correlated to chromatin compaction and transcriptional repression, possibly through an association with HDAC1 (Chakravarthy et al., 2005).

Figure 1.1 Nucleosome structure and the affect of histone variants. (A) Histone octomer containing two copies of H2A-H2B and H3-H4 heterodimers, with their disordered tails protruding from the core particle. (B) Depiction of the core nucleosome particle with certain histone variants that disrupt the DNA dyad resulting in a more open nucleosomal structure. Adapted from Shaytan, Landsman and Panchenko, (2015)
1.1.3 Histone modifications

While the replacement of histones with variants is the most extensive nucleosome modification, histone post-translational modifications [PTMs] are associated with regulating the local chromatin environment. The majority of known post-translations occur on the N-terminal histone tails that protrude from the nucleosome core. Many types of histone modification have been identified on lysine, arginine, glutamine, serine, and threonine residues. The function of these modifications including methylation, acetylation, phosphorylation, and ubiquitination is still not fully understood.

Lysine acetylation on H3 and H4 N-terminal tails correlates with euchromatin and actively transcribed regions. The positive lysine charge is lost upon acetylation, weakening inter-nucleosomal histone and DNA interactions. This mechanism is proposed to directly result in an open chromatin fibre structure. However in vitro studies of repetitive DNA on reconstituted nucleosome arrays showed no significant change to fibre structure due to pan acetylation (Neumann et al., 2009), although globular domain acetylation at the interface of H3/H4 tetramer and H2A/H2B dimer, has been shown to destabilise the histone octamer resulting in more accessible chromatin fibres (Ye et al., 2005).

Histone acetylation occurs at multiple lysines on H3 and H4 including H3 – K9, -K14, -K18, -K56 and H4 –K5, -K8, -K12, -K16, -K91, and is required for their correct assembly. This modification is transferred to histones via histone acetyltransferase enzymes (HATs) and removed by histone deacetylases (HDACs). Bromodomains, a left-handed highly charged pocket formed from four α-helices, recognise histone acetylation and occur
commonly in transcriptional activators and chromatin re-modellers (Filippakopoulos et al., 2012). Maintaining acetylation profiles has an important role in gene regulation and genome stability, where HDAC loss results in aneuploidy. (Hebbes, Thorne and Crane-Robinson, 1988; Dovey et al., 2013).

Methylation also occurs on histone lysines in direct competition with lysine acetylation, in addition to arginine residues. Arginines can be methylated twice while lysines can take three methylation groups and unlike acetylation, methylation does not alter the amino acids charge. Methylation is added to histones in a site-specific manner by different methyltransferases (HMTs); lysine histone methyltransferases (HKMTs) contain the Su(var)3-9, enhancer-of-zeste and trithorax (SET) domain while PRMTs are the class of methyltransferases specific to arginine residues.

Removal of mono-, di-, and tri- methyl groups is tightly regulated. Lysine-specific demethylase 1 (LSD1), uses oxidative demethylation to remove mono- and di- methyl groups (Shi et al., 2004). LSD1 specificity is achieved from the addition of different protein complexes to regulate its binding (Lee et al., 2005). All methylation states including trimethylated lysine residues are demethylated by the large class of jumonji domain containing proteins via Fe(II) and α-ketoglutarate mediated oxidation (Yamane et al., 2006).

Unlike acetylation, histone methylation has been associated with both active and repressive chromatin states. Constitutively inactive chromatin, such as telomeres and pericentric regions are marked with H3K9me3 which in turn recruits heterochromatin protein 1 (HP1) and its own methyl transferase; Suppressor of variegation 3-9 homologue 1 or 2 (Suv39h1/2). This positive
feedback loop maintains the repressed chromatin state throughout the cell cycle (Haldar et al., 2011). In contrast, H3K4me3 is associated with actively transcribed regions along with H3K36 and H3K79 methylation (Martin and Zhang, 2005).

The role of these methylation modifications on gene regulation does not appear to be universal. Confusingly, additional studies have found H3K9me3 at actively transcribed genes and H3K36me2 supressing intragenic transcription initiation (Carrozza et al., 2005; Vakoc et al., 2005). Despite the intricate functions of histone methylation, subsequent loss directly influences genome integrity. Double knockout of the Suv39h isoforms 1/2, responsible for H3K9me3 modification, results in increased rates of polyploidy, meiotic abnormalities, and chromosomal mis-segregation (Peters et al., 2001). These murine models also showed increased incidence of tumour formation and decreased viability, demonstrating the importance of methylation for genome integrity.

Histone methylation is also known to play roles in cellular responses, such as DNA damage, where H3K79me is deposited. DOT1, the HKMT responsible for H3K79me modification, recruits 53BP1 to double strand break sites via its tudor domain which in turn binds to methylated lysines. This process is required for correct repair pathways to proceed. However whether double strand breaks directly affect the local chromatin structure, making it accessible to 53BP1 and DOT1, or if this signalling causes an increase in chromatin accessibility remains unclear (Huyen et al., 2004).

Protein phosphorylation occurs frequently across protein classes and functions, and histone phosphorylation is important for cell cycle regulation.
Histone phosphorylation has been found at serine, threonine and tyrosine residues, for example, H3S10 adjacent to the H3K9 methylation site is phosphorylated by Aurora B as cells begin mitosis (Wei et al., 1999). Phosphorylation at H3S10 causes HP1 proteins, that would normally bind to H3K9me3, to dissociate from the chromatin fibre (Hirota et al., 2005). Without phosphorylation and subsequent loss of HP1 proteins, the loading of condensin is perturbed adversely affecting mitotic condensation and genome integrity (Adams et al., 2001; Giet and Glover, 2001).

Histone modifications can also occur on histone variants such as the phosphorylation of H2A.X at ser139; this happens rapidly after DNA damage and requires the ATM, ATR, and DNA-PK kinases. This phosphorylation of the chromatin fibre occurs in megabase arrays around the double strand break site, where 53BP1 and BRCA1 are subsequently recruited (Soutoglou and Misteli, 2010). After DNA damage has been repaired, H2A.X is dephosphorylated by PP2A and PP4 (Chowdhury et al., 2005) or removed from the chromatin fibre by the FACT complex (Heo et al., 2008). H2A.X knockouts have shown increased genomic instability and cancer prevalence not recoverable in H2A.X rescue experiments with ser139 point mutations (Celeste et al., 2003). While many histone modifications correlate with chromatin fibre states, the changes in fibre compaction after DNA damage provided a tractable system to test the consequences of various histone modifications. DNA damage marks such as phosphorylation of H2AX, H3, and H2B in addition to H2A ubiquitination were not causative however to the compaction state alterations in the chromatin fibre (Fernandez-capetillo, Allis and Nussenzweig, 2004; Kruhlak et al., 2006; Lee et al., 2015; Ui, Nagaura and Yasui, 2015).
In addition to histone modifications functioning in the DNA damage response pathway, recent studies have found that they can also predispose a region to chromosome instability. For instance, the frequency of double strand breaks and translocations is associated with H3K9me3 depletion whereas active marks H3K27ac and H3K4me1 were enriched irrespective of underlying transcription levels (Burman et al., 2015). In this study causality was demonstrated by tethering histone modifiers to a lac operon, altering the density of H3K9me3, H3K27ac, and H3K4me1, which affected break frequency. Despite transcription levels not being affected, H3K9me3 decreased endonuclease accessibility suggesting that a more open chromatin environment is prone to greater instability than more compact H3K9me3 containing regions.

Given the plethora of histone modifications and their enzymatic regulators, histone modifications are often transient. The retention of histone modifications after DNA replication and nucleosome turnover was assessed by capturing nascent chromatin strands (Alabert et al., 2014). In HeLa cells, expelled histones were recycled in the subsequent two nascent DNA strands resulting in a hemi-modified state. Full recovery of histone modifications then occurs within the next cell cycle, except for heterochromatic marks H3K9me3 and H3K27me3 which develop more slowly over multiple cycles. How more rapidly dividing cells, such as embryonic stem cells, maintain heterochromatic domains is an unanswered question.
1.1.4 Bromodomains

As described above, lysine acetylation has been identified as prevalent regulatory post-translation modification that removes the residues positive charge (Kim et al., 2006; Choudhary et al., 2009). Acetyl- ChIP-seq studies have demonstrated a correlation of histone acetylation with actively transcribed genes, with aberrant acetylation and gene expression associated with tumorigenesis (Zhao et al., 2014).

Bromodomains are a highly conserved motif, first identified in the *brahma* gene, that forms and highly charged molecular pocket facilitating protein-protein interactions on acetylated lysines (Haynes et al., 1992). This domain is present in 42 proteins in the human genome with some containing multiple bromodomains or other histone interacting domains (Filippakopoulos, 2012). These proteins display a wide range of functions regulating gene expression. These range from scaffolding roles of large transcriptional complexes and individual catalytic functions including: methyltransferases, HATs, and helicases to function in chromatin organisation (Haynes, 1992; Yamane, 2006; Alsarraj et al., 2013; Bao and Shen, 2014).

The additional domains within bromodomain containing proteins can also affect the binding affinity of the bromodomain pocket. For instance, a single bromodomain typically binds to the histone peptide with the N-terminus at the back of the pocket between the ZA-BC α-helices. This positions the lysine residue at the exit vector on the ZA helix (Filippakopoulos, 2012). An adjacent PHD domain however, as found in TIF1α, engages the N-terminus on H3K4 forcing the bromodomain to bind further down the peptide at
H3K23ac (Tsai et al., 2010). By contrast, the adjacent PHD domain in the BPTF transcription factor differs by having its orientation reversed. When the PHD domain engages with methylated H3K4, the bromodomain orientation is therefore inverted limiting interactions with lysine residues on the same histone tail, and encourages \textit{trans} H4K16 interactions within the same nucleosome (Ruthenburg et al., 2011; Xi et al., 2011).

While bromodomains are highly conserved across the 42 proteins within the human genome and across species, there is a sub family of bromodomain extra terminal [BET] proteins. These contain a C-terminal extra terminal region that allows for selective targeting of these bromodomains from the bromodomain family (Filippakopoulos et al., 2010). The BET protein sub family is comprised of four proteins, Brd2, Brd3, Brd4, and BrdT. Each of these proteins contain two adjacent bromodomains allowing them to bind two acetylated lysine residues such as H4K5 and H4K8 by BrdT (Rousseaux et al., 2009). Whether these adjacent domains bind to lysine residues in \textit{cis} or \textit{trans} histone tails \textit{in vivo} remains an area of discussion within the field.

More recent reports have demonstrated that BET proteins can also recognise non-acetyl lysine modifications including: propionylation, butyrylation, and crotonylation (Tan et al., 2011; Sabari et al., 2015; Goudarzi et al., 2016). The functional role of such non-canonical lysine binding and its effect on BET acetylation interactions has yet to be fully elucidated however.
BET proteins are known to have key regulatory functions. Brd2 and Brd4 are both embryonic lethal in mice, and are known to function in cell cycle progression by regulating transcription of c-myc and other master growth regulatory genes (Houzelstein et al., 2002; Shang et al., 2009; Coudé et al., 2015). Brd3 is known to interact with transcription factor GATA-1 to regulate erythroid specific differentiation genes, whilst BrdT is a testis specific transcription factor with similar functions to Brd4. Loss of BrdT results in aberrant testis development resulting in sterile mice (Shang et al., 2007; Lamonica et al., 2011).

The canonical transcriptional regulatory mechanism of BET proteins revolves around the recruitment of the p-TEFb complex (CDK9 and cyclin T) to transcriptional start sites, facilitating paused RNA polII phosphorylation and transcriptional elongation (Kyoo Jang et al., 2005; Yang et al., 2005; Itzen et al., 2014). Given the fundamental functions of these proteins for efficient transcription and faithful cell cycle progression, their binding profiles vary significantly between cell types. The regulatory mechanism behind these different binding profiles has yet to be fully understood and is currently an area of significant interest.
Additionally bromodomain protein mutations commonly occur in cancer, likely due to their key role in transcription and proliferation. In Paediatric cancers, for instance, 21 of the 42 bromodomain containing proteins have increased mutation frequencies. How these mutations effect cellular phenotypes have yet to be fully explored however. Bromodomain proteins are also common sites of translocations and gene fusions in cancer. NUT midline carcinoma, for example, is caused by the fusion of Brd3 or Brd4 to nuclear protein in the testis (NUTM1). NUT proteins normally function to recruit HAT enzymes including CREBBP and P300, acetylating the surrounding histones. When fused to a BET protein that in turn recognises histone acetylation, this results in unregulated expanding regions of hyperacetylation only limited by TAD boundaries resulting in this disease (Haack et al., 2009; Alekseyenko et al., 2015). Understanding the function and regulation of BET proteins therefore is a key question in both developmental and therapeutic fields.
1.1.5 Chromatin remodelling complexes

A diverse range of proteins aside from histones bind chromatin in vivo. Chromatin re-modellers alter nucleosome frequency or composition where they act at a local level causing small scale disruptions to the chromatin fibre and changing the accessibility of the underlying DNA. With such a fundamental role chromatin re-modellers are found in many chromatin dependent processes, such as replication, cell cycle progression, DNA damage repair and transcription. The ATP dependent active domains of these re-modellers are split into four classes: SWI/SNF, ISWI, INO80, and CHD.

SWI/SNF re-modellers are found in large complexes with other polypeptides in two different classes: BAF (BRG1 associated factors) and PBAF (Polybromo associated factor). In humans, BAF complexes contain both ATPase subunits, BRM and BRG1. PBAF by contrast only contains BRG1, but sharing many other bromo associated core factors with BAF complexes (Mohrmann and Verrijzer, 2005). SWI/SNF specificity is regulated by varying the DNA binding domain and bromodomain composition of subunits, allowing loci and tissue specificity via nucleotide sequence and the nucleosome acetylation state (Shen et al., 2007; Dechassa et al., 2012). SWI/SNF function is important for genome regulation, and components have an increased mutation rate in cancer. PBAF component BAF180 is recruited to DNA damage sites resulting in silencing of the surrounding region (Kakarougkas et al., 2014). SWI/SNF complexes also have important functions in sister chromatin cohesion and decatenation. Specifically, loss of SWI/SNF components results in an increase in cytological chromosome breaks and lagging chromosomes in mitosis respectively (Dykhuizen et al., 2013; Brownlee et al., 2014).
The ISWI class of re-modellers incorporate SNF2H or SNF2L ATPase’s. They are associated with a diverse range of functions, both repressing and activating target loci. The ISWI complex NURF is recruited to promoters by the GAGA transcription factor, where it alters nucleosome spacing to facilitate transcription (Mizuguchi et al., 1997; Xiao et al., 2001). Studies investigating the distribution of core ISWI catalytic subunits in Drosophila found that ISWI rarely correlated with RNA polymerase II distribution on polytene chromosomes, suggesting it has a more general role at transcriptionally inactive genomic regions (Deuring et al., 2000). Furthermore in yeast, ISWI homologues reposition nucleosomes at promoters, making them nuclease-inaccessible and repressing gene expression (Goldmark et al., 2000). ISWI also functions in the DNA damage response pathway. The ACF and CHRAC complex subunit ACF-1 interacts with HP1 proteins and binds to DNA double strand breaks similarly to phosphorylated H2A.X facilitating NHEJ break repair (Eskeland, Eberharter and Imhof, 2007; Luijsterburg et al., 2009).
INO80 chromatin re-modellers are characterised by having a split ATPase domain across two subunits. This ATPase module associates with RuvB-like helicase proteins, known to bind replication forks and holiday junctions in vitro. (Iwasaki and Takahagi, 1991; Shen et al., 2000). These complexes were shown to regulate H2A.Z and phospho-H2A.X histone exchange throughout the genome and at DNA damage sites (Papamichos-chronakis et al., 2012; Bao and Shen, 2014). Loss of INO80 and redistribution of H2A.Z causes fork collapse slowing S-phase progression (Hur et al., 2010) whilst loss of INO80 at DNA damage foci disrupts the 5’-3’ resection of the DNA strands during homologous recombination (Gospodinov et al., 2011). Histone acetyltransferase TIP60, a component of the INO80 complex, is required to open chromatin for DNA repair and to subsequently remove phospho-H2A.X to restore the basal chromatin state (Kusch et al., 2004).

In addition to acetyl recruiting poly bromodomain re-modellers such as BAF180 (Porter and Dykhuizen, 2017), chromo helicase domain (CHD) complexes are characterised by their ability to bind methylated histones. The nucleosome remodelling and deacetylase complex NuRD is the only CHD complex currently thought to be recruited via transcriptional regulators. The active subunit CHD3/4 with HDAC1/2 promotes transcriptional repression of the Hox locus through its interaction with hunchback in Drosophila (Kehle et al., 1998; Zhang et al., 1998). NuRD’s interaction with the ELKF transcription factors is mediated by the addition of small ubiquitin-like modifiers (SUMOs), resulting in KAP-1 target transcriptional repression (Siatecka, Xue and Bieker, 2007). In addition to gene regulation, CHD3 – NuRD complexes are released from heterochromatin after DNA damage resulting in chromatin fibre disruptions (Goodarzi, Kurka and Jeggo, 2011). CHD recruitment specificity via its chromodomain to methylated histones,
however, remains controversial and chromodomain deletions of CHD1 in *Drosophila* do not affect its distribution and can bind H3K4 peptides independently of the methylation state (Morettini *et al.*, 2011).

Chromatin re-modellers play an important role in facilitating the dynamic changes in chromatin fibre structure required throughout the cell cycle, kinase signalling responses and gene regulation. Understanding their mechanical properties in manipulating the chromatin fibre is an area of interest within the field; new cryo-electron microscopy approaches are providing significant new insight (Aramayo *et al.*, 2018). However, our limited knowledge of chromatin fibre structure still limits our understanding of how fibres are manipulated by remodelling complexes.
1.1.6 Topoisomerases

In addition to manipulating nucleosomes to alter chromatin fibre structure, abnormal DNA topology and catenation can affect the overlying chromatin structure. The topoisomerase family exhibit the capability to resolve these exotic DNA tertiary structures by inducing single or double stranded breaks (Charvin, Bensimon and Croquette, 2003). Type 1 topoisomerases (Top1) introduce single strand breaks and are subdivided into A/B depending on whether they bind to the 5’ or 3’ strand respectively. Top1A enzymes allow one DNA strand to pass, bridged by the enzyme, relaxing negative supercoiled DNA (Dekker et al., 2002). Top1B in contrast allows the DNA strands to rotate independently of each other in a torsion dependent manner, releasing either positive or negative supercoils (Koster et al., 2005; Taneja et al., 2007). Type 2 topoisomerases (Top2) are also subdivided into A/B on their molecular structure but have similar mechanisms of action. Top2 releases topological stress by inducing double stranded DNA breaks facilitated by ATP-dependent reverse gyrase activity (Roca and Wang, 1992; Berger et al., 1996).
Many biological processes induce mechanical stress on chromatin topology including, transcription, replication, and anaphase. During transcription RNA polymerase over-winds DNA downstream inducing positive supercoils, whilst negative supercoils are formed upstream (Liu and Wang, 1987; Giaever and Wang, 1988). This produces a positive or negative extra turn in the DNA molecule every 10 bp of transcription, making topoisomerase activity essential for the transcription of long genes, and prevents exotic DNA structures such as plectonemes and cruciforms (Cook et al., 1992). Mutations in Top1 in autistic patients appear to preferentially affect transcripts >200 kb, indicating the important function of topoisomerases (King et al., 2013). Transcription-dependent supercoiling from has been shown to stall replication forks, but normal Top1 activity functions to resolve these conflicts, facilitating replication of active genomic regions (Tuduri et al., 2009).

Top2 enzymes are proposed to resolve the positive or negative supercoils across replication regions. Converging replication forks can amplify this topological stress slowing replication in the absence of Top2 (Baxter and Diffley, 2008). In addition to this function, Top2 has been implicated in chromatin condensation (Adachi, Luke and Laemmli, 1991). In metaphase Top2 forms a structural axis the length of the chromosome required for chromatid core separation (Giménez-Abián et al., 1995), whilst Top2 loss reduces intra-chromatid fidelity resulting in entangled chromosome arms (Uemura et al., 1987; Spence et al., 2007). The consequences of a loss of chromatid fidelity results in multiple aberrant mitotic phenotypes, prevents cell cycle progression and induces cell death (Carpenter and Porter, 2004). The exact role of Top2 at mitotic chromosomes remains unclear, along with their role within high order chromatin structures.
1.1.7 Cohesin and Condensin

Structural maintenance of chromosome (SMC) proteins, are found in the highly conserved condensin and cohesin complexes. These complexes function in the modulation of chromosome structure and are required for transcription, mitotic condensation, and chromatid separation. SMC proteins have large coiled arms of alpha helices spanning 65 nm, with globular heads at either end. One of these domains interacts with its SMC partner forming a molecular hinge to the complex (Haering et al., 2002). The diametrically free head domains of this SMC heterodimer interact with a kleisin-type component, in order to close the molecular loop in an ATP-dependent manner (Hu et al., 2011). Due to this large ring like structure SMC complexes are thought to associate with chromatin topologically rather than a DNA sequence mediated interaction (Murayama and Uhlmann, 2014).

Cohesin complexes contain an SMC1/SMC3 heterodimer closed by RAD21, with chromosome loading facilitated by STAG (Tóth et al., 1999; Hu et al., 2011). Mammalian condensin has a SMC2/SMC4 heterodimer, and two separate groups of subunits. Condensin I is closed by CAP-H with CAP-D2 and CAP-G (Hirano, Kobayashi and Hirano, 1997; Schmiesing et al., 2000). Condensin II contains CAP-H2, CAP-D2 and CAP-G comparatively (Ono et al., 2003; Yeong et al., 2003).
Despite their striking structural similarities, the molecular functions of cohesin and condensin are distinct. Cohesin maintains sister chromatin cohesion after replication to allow faithful separation upon its cleavage during anaphase (Uhlmann, Lottspeich and Nasmyth, 1999). Cohesin is initially loaded onto chromatin in an ATP-dependent manner during G1 by SMC loader NIPBL (Ciosk et al., 2000). Throughout G1 cohesin levels equilibrate via its constant unloading via WAPL and PDS5 (Tedeschi et al., 2013). During replication, the cohesin ring structure is stabilised by SMC3 acetylation mediated by Sororin which prevents WAPL-cohesin removal (Hou and Zou, 2005; Nishiyama et al., 2010). In conditional WAPL knock out cells, cohesin unloading is also prevented resulting in vermicelli like chromatin condensation in G1 and abnormal chromosome segregation during mitosis (Tedeschi et al., 2013). Regulation of cohesin loading and stabilisation therefore has a fundamental architectural role in chromatin organisation throughout the cell cycle.

In mitosis, for WAPL to access cohesin complexes and allow sister chromatin separation, Aurora B or CDK1 phosphorylates Sororin expelling it from cohesin complex, (Dreier, Bekier and Taylor, 2011). This initially occurs along the chromosome arms during prophase as the centromere is protected from phosphorylation by Shugoshin recruited phosphatases (Meppelink et al., 2015). During anaphase, cleavage of centromeric cohesin occurs by separase (Uhlmann, Lottspeich and Nasmyth, 1999). In order for cohesin complexes to be reloaded in the following cell cycle, HDAC8 is required to deacetylate SMC3 (Xiong, Lu and Gerton, 2010; Deardorff et al., 2012).
Cohesin has also been implicated in modulating large-scale chromatin structure. Cohesin chromatin loading correlates with CTCF sites at structural chromatin boundaries (Parelho et al., 2008). This has led to the proposal of cohesin loops linking chromatin fibres in the same manner as sister chromatids but in trans across a chromosome. Recent Hi-C studies in cohesin knock out cells results in the loss of all loop domains (Rao et al., 2017). Despite such stark changes in large-scale chromatin organisation, transcriptional regulation is only modestly affected. This suggests that while cohesin complexes help organise chromatin throughout the nucleus they do not inherently regulate the accessibility of the underlying genes (Schwarzer et al., 2017).

It is maybe not surprising then that mutations in cohesin subunits are not lethal, albeit with severe pathology. Patients with cohesin mutations, such as Cornelia de Lange Syndrome (CdLS), exhibit cognitive impairment, limb defects, and delayed development. However rather than mitotic defects, CdLS cellular defects appear to be transcriptional, suggesting that the minor regulatory role of cohesin on transcription is an essential function in development outside of mitotic division (Liu et al., 2009). Indeed the loss of a single boundary element can result in aberrant enhancer activity and transcription in nearby genes (Lupiáñez et al., 2015).
Condensin’s primary role is to compact chromosomes in the early stages of mitosis and resolve sister chromatid cohesion in cooperation with Top2 (Coelho, Queiroz-Machado and Sunkel, 2003; Hirota et al., 2004). However, condensins also have regulatory roles within interphase. Hi-C data indicates that condensin complexes co-localise with transcription factor IIIC complexes (TFIIIC) to form strong TAD boundaries during interphase that support constitutively active gene cluster transcription (Yuen, Slaughter and Gerton, 2017).

During mitotic compaction condensin I facilitates lateral chromosome compaction, while condensin II is required for longitudinal compaction across the length of the chromosome (Green et al., 2012).

Polymer modelling of Capture-C experiments first suggested the formation of consecutive chromatin loops 80-120 kb in mitotic chromatin fibres (Naumova et al., 2013). This has led to the proposal that condensin modulates these large chromatin looping domains (Skoko et al., 2006; Alipour and Marko, 2012). Once formed, these loops then undergo axial compression mediated by Top2 (Coelho, Queiroz-Machado and Sunkel, 2003). Condensin’s role in chromatin condensation is however still questioned, as chromatin compaction is not completely abrogated in condensin or Top2 absence (Hudson et al., 2003; Carpenter and Porter, 2004; Houlard et al., 2015). This has led to the contrasting view that condensins regulate higher order chromosome architecture rather than the underlying chromatin fibre compaction state (Vagnarelli et al., 2006).
Although Condensin I and II have contrasting roles, they function cooperatively for correct mitotic cytological compaction. Detailed experiments in *Xenopus* extracts demonstrated that altered ratios of condensin I and II resulted in longer and thinner chromosomes (Shintomi and Hirano, 2011). Primary condensin II mediated cytological condensation is thought to be essential for correct mitotic chromosome formation as condensin I is cytoplasmic until the breakdown of the nuclear envelope (Gerlich *et al.*, 2006).

Similar to cohesin loading, condensin II loading is stabilised during S-phase. After replication, condensin II stabilisation mediates the formation of sister axes functioning in faithful resolution of sister chromatids (Ono, Yamashita and Hirano, 2013). Once the nuclear envelope is dissolved, condensin I then associates along the chromosome in an alternating distribution with condensin II (Ono *et al.*, 2004). Recent seminal studies have demonstrated the spiral loading dynamics of condensin II complexes to form the axial core of metaphase chromosomes (Gibcus *et al.*, 2018). This spiral core supports the formation of condensin I mediated nested loops resulting in the characteristic late prometaphase chromosomes.

Recent studies have also suggested that condensin II interacts with holiday junction recognition protein (HJURP) and is required for correct centromeric deposition of CENP-A and centromere function (Barnhart-Dailey *et al.*, 2017). In conjunction with Gibcus’s, (2018) work suggesting condensin II loading only occurring at the chromosome core, this would provide a mechanism for kinetochore attachment to each chromosome’s for proper segregation.
1.2 Chromatin Structure

Chromatin structure is both dynamic and diverse making studying its structure a challenging goal. In nuclei, human interphase chromatin is not discernible by traditional microscopy techniques. Recent work has coupled DNA staining with polymerisation of diaminobenzidine (DAB) for electron microscopy to study chromatin in structure inside nuclei (Ou et al., 2017). The validity of such techniques to broadly study chromatin structure is still debated however, as there may be a bias in the dye (DRAQ5) DNA binding distribution. DRAQ5 may preferentially bind to more open euchromatic regions or be unable to come into contact with DAB molecule when bound to heterochromatic DNA.

Unlike humans, mouse chromatin has distinct chromatin structures observable by traditional microscopy methods. Observable heterochromatic chromocenters form in many species including mice, although the underlying structure is still unresolvable. *In vitro* studies of chromatin via electron microscopy while revealing characteristics of fibre structure are limited in their applicability to the complexity of *in vivo* chromatin. For instance understanding how current fixation methods might affect fibre structure, in electron microscopy methodology remains an important question.
1.2.1 Nucleosome arrays and the 30-nM fibre

The base unit of eukaryotic chromatin contains, a nucleosome core particle of a H3:H4 tetramer with two H2A:H2B dimers bound to it. Wrapped twice in 147 base pairs of DNA, these nucleosome core particles are stabilised by histone H1 binding on the dyad and connected by 10-100 bp “linker” DNA (Holde, 1989). The exact position of H1 is still debated, with one model claiming the globular domain lies on top of the dyad interacting with both DNA linker strands (Allan et al., 1980; Hussain et al., 2010). Alternatively the globular domain is removed from the dyad and interacts with only one linker DNA strand (Pruss et al., 1996; Brown, Izard and Misteli, 2006). More recent studies have shown that linker histone isoforms H1 and H5 differ in their binding positions due to altered DNA-contacting residues in Drosophila and chicken respectively (Zhou et al., 2016). Off dyad binding has also been demonstrated in the human linker isoform H1.4 (Song et al., 2014). Additionally canonical H1 has recently been shown to bind at the dyad but primarily associating with a single DNA linker, suggesting an asymmetrical nature to the nucleosome (Bednar et al., 2017).
While our knowledge of nucleosome composition is well defined, how multiple nucleosomes are folded into a fibre with a width of 30-nm is still hotly debated. Multiple models have been advocated; the solenoid model with a helical array, the zig-zag model of alternating nucleosome stacking, and an irregular model with varying nucleosome spacing and organisation. Recent advances in super resolution microscopy in addition to electron microscopy techniques and small angle x-ray diffraction have been used over the past 50 years to discern fibre structure (Gall, 1966; Sperling and Tardieu, 1976; Ricci et al., 2015). While investigating in vitro reconstituted chromatin has been possible, the structure of chromatin fibres within nuclei has remained elusive, only defined as a dense mass.

Despite such debate, chromatin within a living cell is required to be dynamic and malleable, unlikely to result from a homogenous 30-nm fibre structure. More likely is a heteromorphic fibre of multiple models, with more compact or disrupted loci dependent on the genomic region (Grigoryev et al., 2009). This is likely due to the underlying transcriptional state with constitutively inactive regions having regular nucleosomes and forming a more uniform 30-nm fibre structure, whereas, global conformation is less regular and interspersed with many disruptions (Gilbert and Allan, 2001). Chromatin remodelers alter nucleosome positioning allowing transcription factor binding, DNA replication, and repair to occur. Therefore one can see how in the dynamic nature of a living, dividing cell responding to environmental stimuli, fibre disruptions may occur resulting in a heteromorphic fibre structure. These disruptions to nucleosome occupancy can be investigated through the accessibility of the DNA to nuclease digestion (Allan et al., 2012).
Figure 1.2 Overview of the different orders of chromatin organisation. (A) 147 bp of the helical DNA structure is wrapped around an octomer of histone proteins to form a nucleosome. Sequential histone deposition results in a ‘beads on a string’ like 10 nm fibre, while inter-nucleosome interactions generates a chromatin fibre in the order of 30 nm. The compaction mechanisms above 30 nm have not been fully resolved, however chromatin forms distinct large-scale structures in interphase that are then further compacted into metaphase chromosomes. (B) DRAQ5 staining in interphase demonstrates the accumulation of chromatin at the nuclear periphery. (C) Hi-C modelling throughout mitosis suggests large-scale loop structures emanating from a spiral axial core on condensin molecules (red). Resources collated from Alberts, (1994); Ou, (2017); and Gibcus, (2018)
1.2.2 Large-scale chromatin structure

Beyond the 30-nm fibre, chromatin fibres are organised into large-scale chromatin structures. Methods to investigate large-scale structures are currently limited, and detailed understanding is lacking, outside the probable formation of chromatin loops. Domains thought to correspond to ‘accessible’ and ‘compact’ regions can be differentiated by their AT:GC nucleotide ratio, histone modification profiles and subsequent bound proteins. Historically this has been segmented into gene rich euchromatin, and gene poor heterochromatin, based on the intensity of intercalating dyes in interphase. Interphase euchromatin has a lower intensity of intercalating dyes than heterochromatin and therefore is perceived as more open, although this could be attributed to DAPI sequence bias. During metaphase however chromatin is uniformly dense suggesting that de-compaction post mitosis must be targeted.

Recent genome wide studies of transcription and modification states has provided greater stratification into five different classifications: 1- constitutively active regions, 2- tissue specific active loci, 3- silenced developmental and differentiation loci, 4- repressed gene rich regions, and 5- constitutive silenced satellite and repetitive elements (Steensel, 2011). Regions 1 and 2 contain the actively transcribed regions of the genome characterized by their enrichment in histone acetylation. Due to transcriptional activity within these loci, they are expected to contain more disruptions to the chromatin fibre, resulting in a more flexible open structure to facilitate transcription. Transcriptionally repressed genes and repetitive elements in the other classes by contrast will have a more ordered compact higher order structure.
Living cells do however maintain a functional need to access these more compact regions for biological processes such as replication and DNA damage repair mediated by chromatin re-modellers. The inherent property of the compact higher order structure does however reduce the accessibility of the underlying DNA when compared to a disrupted fibre. This is discernible by these regions being late replicating and responding more slowly to DNA damage (Keohane et al., 1996; Sadoni et al., 1999).
1.2.3 Higher order structure methods and TADs

Chromosome conformation capture (3C) and fluorescence in situ hybridization (FISH) based methods are the two most widely adopted techniques to study chromatin higher order structure (Bickmore and Van Steensel, 2013). FISH involves the hybridization of fluorescent probes with specificity to individual loci up to entire chromosomes. Chromosome conformation capture by contrast scores the frequency of a DNA fragment contacting with another that can be multiplexed by next-generation sequencing across the entire genome. This relies on “fixing” the chromatin structure with formaldehyde, fragmenting and ligating proximal DNA fragments, before the PCR or sequencing output. These techniques have complimentary technical methodologies. FISH benefits as a single cell technique but with resolution limited by microscopy methods, while 3C methods have base pair resolution, but is limited as it is population average data and has the potential bias input by the fixation technique.

The prevalence of 3C-based experiments has led to adoption of the topological associating domains (TADs) concept. This suggests that the human genome is divided into almost ten thousand loops/TADs on average 185 kbp in size (Rao et al., 2014). These TADs prevent external chromatin interactions, correlating with histone modification profiles and replication timing (Pope et al., 2014; Huang et al., 2015). The functional relevance of TADs to genome regulation is nonetheless still under debate, as TAD profiles are not affected by cell type. However translocation frequency in B cells correlates with 3C interaction frequencies, demonstrating a functional role to interaction frequency (Y. Zhang et al., 2012).
Validation of 3C results using FISH has however had varied success. The confinement of intra-TAD chromatin interactions and organisation identified by 3C methods was confirmed by intra-TAD FISH probes having a physically closer association within the nucleus when compared to similar probe genomic distances between TADs (Nora et al., 2012). By contrast a comprehensive study at the HoxD locus demonstrated varying success of cross validation in different developmental stages (Williamson et al., 2014), suggesting that the two techniques might report on different levels of organisation.

However, these techniques have facilitated a large step in our understanding of higher order chromatin structure, despite their limitations. The advancement of these methodologies currently in process hopes to resolve the current conflicting experimental data. 3C techniques have been combined with sequence capture methods to allow deeper sequencing of defined genomic regions increasing the techniques resolution. Additionally, the Mistelli lab has addressed the throughput limitations of FISH experiments (Shachar et al., 2015). Subsequent studies comparing Hi-C datasets to high throughput FISH has demonstrated a common independent allele in addition to population heterogeneity that was not detected by Hi-C (Finn et al., 2017). This suggests that cell population based techniques have limited scope to investigate chromatin structure.

The static nature of our current understanding of genomic interactions will soon be addressed by the adaption of the CRISPR-Cas9 system. A recent study demonstrated the use of modified guide RNAs to achieve live cell imaging of non-repetitive loci, demonstrating a significant advantage to aid our understanding of chromatin fibre dynamics (Qin et al., 2017).
1.3 Nuclear environment

Chromatin in vivo is found within the nucleus of a eukaryotic cell, determining the spatial environment for chromatin organisation. The nuclear position of a locus has a functional role in chromatin organisation, affecting chromatin structure, transcriptional state, and replication timing. The nuclear spatial orientation of a locus is unfixed and dynamic. Throughout differentiation many loci alter their nuclear position changing their functional properties (Peric-hupkes et al., 2010).

Nuclear positioning however is not tightly defined and varies between cells, although intra-chromosomal long range interactions are tightly regulated (Chambeyron and Bickmore, 2004). Despite undefined specific 3D spatial orientations, loci’s radial 2D distribution appears to be conserved throughout the genome. In mammalian cells the nuclear periphery generally contains gene poor regions and heterochromatic elements, while active genes usually have a more central position. In contrast, centromeres are generally located at the nuclear periphery, while telomeres are equally distributed throughout the nucleus in differentiated cells (Gilchrist et al., 2004; Wiblin et al., 2005).

The nuclear membrane, which separates the nucleus from the cytoplasm, is composed of a lipid bilayer with nuclear pore complexes facilitating shuttling. Lamin proteins are transported into the nucleus where they accumulate around the inner nuclear membrane forming the nuclear periphery once they are phosphorylated and post-translationally mature (Henekes et al, 1993; Kilic et al, 1999). Genomic regions at the nuclear periphery form lamina associated domains (LADs) between 100 kbp and 10
Mbp in size. Nuclear membrane components; the lamin B receptor, emerin, BAF, LAP2α/β, and SUN proteins facilitate this association. Constitutive and facultative LADs are found within the genome. Facultative LADs tend to be the silenced developmental and differentiated genomic regions that differ between cell types, whereas constitutive LADs are universal. Signalling pathways and chromatin modifiers interact with nuclear lamina proteins to regulate nuclear periphery heterochromatin histone acetylation, H3K9me3, and subsequent HP1α/β association (Oca, Andreassen and Wilson, 2011; Solovei et al., 2011). Importantly, Solovei (2011) showed that the lamin B receptor alone is required to tether heterochromatin to the nuclear periphery and is required for Lamin A and Lamin C mediated tethering.

Heterochromatic domains are also recruited to the periphery of the nucleolus in a mechanism requiring nuclear lamins. Absence of Lamin B1 leads to dissolution of nucleoli (Martin et al., 2009). LADs and nucleolar associated domains (NADs) are often flanked by insulators such as CTCF which tether the ends of the domains to the nuclear periphery in a lamin A dependent manner (Ottaviani et al., 2009).
Premature lamin A that has not undergone the required farnesylation, directly binds to HP1α in addition to lamina bridging proteins LAP2α and BAF causing their accumulation at the nuclear periphery (Mattioli et al., 2007). The interaction with HP1α is lost when prelamin A is farnesylated. HP1α disassociation from prelamin A-LAP2α complex, is thought to be a key regulator of chromatin conformation and transcription in myoblast differentiation (Lattanzi et al., 2007; Capanni et al., 2008). LAP2α lamin-chromatin interaction loci are regulated by nucleosomal HMGN5 regulating the level of heterochromatin condensation and mobility (Zhang et al., 2013).

A series of mutations have been identified in lamin proteins associated with a wide range of phenotypes including muscular dystrophies, neuropathies, metabolic disorders, and premature ageing, together classed as laminopathies (Peters, John et al., 1998; Bonne et al., 1999). Lamin knockout models only show defects postnatally continuing the idea that the nuclear periphery functions in terminal differentiation. The rare and fatal Hutchinson-Gilford progeria syndrome (HGPS) laminopathy presents a premature ageing phenotype at 12 months and has received the most attention. HGPS patients have an LMNA mutation, disrupting the nuclear periphery organisation, and altered histone modification distributions. In more common laminopathies such as skeletal muscular dystrophy only rare patient samples found any evidence of abnormal nuclear envelope and heterochromatin structure and this effect was heterogeneous throughout the tissue sample (Sabatelli et al., 2001; Sewry et al., 2001). This less severe disruption to the nuclear periphery likely leads to the less severe phenotype and increased survival.
1.3.1 Chromosome territories

Chromosomes are de-compacted from mitosis to interphase, and occupy distinct 3D spatial volumes. This was first demonstrated by hybridising large numbers of specific probes across a specific chromosome, in fluorescence in situ hybridisation (FISH), to visualise a whole chromosome within the nucleus (Cremer et al., 1988). The radial distribution of a chromosome is determined by its gene density, with gene poor chromosomes more likely to be associated with the nuclear periphery (Boyle et al., 2001). Comparison of chromosomes 18 and 19, which have similar genomic sizes, maintain significantly different nuclear volumes and position. Gene rich chromosome 19 is found more centrally and has a larger nuclear volume than gene poor chromosome 18 in lymphoblastoid and fibroblast cell types (Croft et al., 1999). Chromosome territories are not fixed, however, and differ in a tissue specific manner, with more closely related cells maintaining more similar nuclear territories in mice (Parada, Mcqueen and Misteli, 2004). Unlike mice where every chromosome is acrocentric, humans have 5 acrocentric chromosomes containing the tandem repeats of the ribosomal genes on their short arms. These acrocentric chromosomes cluster around the nucleolus to allow processing of the ribosomal RNA.

The intra-chromosomal radial distribution within a nuclear territory is also affected by gene density. Again gene rich regions are more central than gene deserts that are more situated nearer the periphery (Boyle, Rodesch and Bickmore, 2011). This nuclear organisation allows transcriptionally silent chromatin to be packaged more densely at the periphery while the less dense central volume is occupied by transcriptionally active loci. This less crowded central volume increases the accessibility of complexes, and metabolic factors
required to facilitate transcription allowing for some tissue specific required expression (Moen and Johnson, 2003). Given our limited understanding of chromatin fibre structure, the more discriminating components behind chromatin territories currently remains unascertained.

Figure 1.3 Nuclear radial distribution of HSA18 and HSA19 chromosomes in hESC’s (A) hES cells DAPI stained nuclei, with hybridised HSA18 and HSA19 chromosome paints. Scale bar is 5 µm. (B) The radial distribution of HSA18 or HSA19 chromosome paints from de-convolved 2D images, binned into pentiles. N=50. Adapted from Wiblin, (2005)
1.3.2 Nuclear Scaffolds

Whether chromatin is suspended within the nucleus or attached to a nuclear matrix has been widely debated since the 1980s. Electron microscopy experiments demonstrated the presence of irregular fibres connected to the lamina at the nuclear periphery (Nickerson et al., 1997). This scaffold is lost after RNase but not DNase digestion suggesting an RNA component to its structure or regulation. However these structures have never been observed in live cells. (Craig et al., 1997) demonstrated that the genomic regions associated with the scaffold were enriched for satellite repeats associated with cytological gene poor G bands. The nuclear matrix proteome was investigated by 2D electrophoresis after removing soluble proteins, lipids, cytoskeleton components, and chromatin from samples consecutively (Fey, Wan and Penman, 1984). Over 300 proteins have subsequently been identified as associating with this scaffold (Mika and Rost, 2005). The main classes of proteins identified were lamins, nuclear mitotic apparatus protein, and heterogeneous nuclear ribonucleoproteins (hnRNPs). The hnRNP A1 and B1 were proposed to regulate the nuclear matrix. This remained controversial due to their scarce abundance on the nuclear matrix filaments and inconsistent evidence, leading to the adoption of more dynamic models for nuclear organisation. Despite this concept of adoption remaining peripheral, recent studies have championed non-coding RNAs as pertaining nuclear scaffold functions.
The most defined role for a ncRNA on chromatin structure is XIST. Found on the X chromosome, this 15-17 kb long non-coding RNA is required for X chromosome compaction and silencing (Penny et al., 1996). Recent super resolution experiments suggest that XIST localises to around 200 foci on average 1 Mb apart along the X chromosome (Smeets et al., 2014; Sunwoo, Wu and Lee, 2015). The interaction of XIST to the inactive X remains even in conditions that strip histones from the chromatin, suggesting a tight association with the underlying DNA (Clemson et al., 1996). This tight association has made identifying protein interactors difficult, however an elegant study coupled RNA antisense purification with SILAC to identify functional interactions with SHARP, SAF-A, and LBR (McHugh et al., 2015). SHARP, a subunit of the SMRT co-repressor, recruits HDAC3 and is required for the deposition of H3K27me3 by PRC2 along the X chromosome (You et al., 2013). The XIST interaction with the lamin B receptor (LBR) has recently been supported by studies demonstrating a causative role for XIST RNA in X chromosome tethering to the nuclear lamina (Chen et al., 2016).

More recent studies identified highly abundant interspersed repetitive elements, such as LINE-1 and CoT-1 associating with less dense euchromatic regions and fractionate with the nuclear scaffold components. Loss of these repetitive elements also causes chromatin condensation (Hall, Lisa et al., 2014). Additionally long non-coding RNA HOTAIR interacts with polycomb component PRC2, targeting H3K27me3 mediated gene silencing at certain loci (Rinn, John et al., 2007). These examples demonstrate that the interplay between non-coding RNA and their binding partners is likely crucial in their function in regulation of chromatin structure and transcription.
Recent papers have begun to contest the direct tethering of chromatin repressors by RNA, however. *In vitro* biochemical analysis by Wang *et al.*, (2017) suggested that PRC2 has robust DNA binding affinity towards methylated GC rich linker DNA, and that RNA acts as a competitive inhibitor to this DNA binding sequestering PRC2 away from the chromatin. This confirms the *in vivo* studies demonstrating PRC2 binding to nascent RNAs (Kaneko *et al.*, 2013). The nucleosome-depleted regions at active promoters would therefore enhance PRC2 binding, suggesting that RNA mediated sequestering of PRC2 from actively transcribed genes as a fundamental process of constitutive gene expression (Yuan *et al.*, 2005; Schones *et al.*, 2008; Helbo *et al.*, 2017). Additionally, the direct tethering of PRC2 by HOTAIR has recently been questioned. Portoso *et al.*, (2017) demonstrated that HOTAIR recruitment alone was required for gene silencing, and that PRC2 recruitment is a consequence of the loss of nascent RNA after transcriptional repression.

An example of RNA binding proteins regulating chromatin structure was recently demonstrated by the hnRNP protein, SAF-A. This protein was identified as being associated with the nuclear matrix (Romig *et al.*, 1992). The function of SAF-A remains unclear but has identified roles in DNA repair, kinetochore assembly and gene splicing (Ma *et al.*, 2011; Xiao *et al.*, 2012; Britton *et al.*, 2014). A recent comprehensive study demonstrated that SAF-A formed transcriptionally dependent large oligomers that maintain euchromatic chromatin structure. Additionally SAF-A loss resulted in the accumulation of phospho-H2AX, implicating SAF-A in genome stability (Nozawa *et al.*, 2017).
1.4 Chromatin structure and gene regulation

The first evidence for chromatin de-condensation upon gene activation was the accessibility of nucleases to digest active and inactive genes (Gottesfeld, Murphy and Bonner, 1975; Bloom and Anderson, 1979). This was first observed directly when transcriptional activators were tethered to regions labelled with GFP in vivo (Tumbar, Sudlow and Belmont, 1999; Tsukamoto et al., 2000). Although this was a synthetic setting, FISH experiments also demonstrated chromatin de-condensation at the endogenous Hoxb cluster (Chambeyron and Bickmore, 2004) after activation. In addition to cytological de-condensation, gene activation is now associated with hyperacetylation, hypomethylation, DNaseI sensitivity, and loss of linker histones, indicative of generating more accessible chromatin fibres.

The basal state and level of de-compaction is dependent of the genomic loci. A heterochromatic transgene spanning 90 Mbs de-condensed 30 fold, while a 375 kb region of the major histocompatibility complex de-condensed less than 2 fold from a more open basal state (Tumbar, Sudlow and Belmont, 1999; Müller et al., 2004). The highest level of cytological de-condensation has been observed at highly active genes such as 7S RNA and ribosomal RNA genes theoretically de-condensing to the level of the 10 nm fibre (Daneholt et al., 1982; González-Melendi et al., 2001). Experiments using sucrose sedimentation techniques demonstrated that chicken erythrocyte β-globin gene had a more disrupted chromatin fibre upon activation (Kimura et al., 1983). This was later shown to be due to the loss of a nucleosome at a DNaseI
hypersensitive site, creating a disruption, thus fibre de-condensation upon gene activation has yet to be directly demonstrated (Caplan et al., 1987). However the presence of a polymerase molecule on a chromatin fibre is associated with a temporary de-compaction from the 30 nm chromatin fibre state (Andersson, Bjorkroth and Daneholt, 1984). Additionally induced gene activation on plasmids in yeast sediment more slowly, indicative of a more disrupted chromatin fibre (Kim and Clark, 2002). Furthermore, the Bickmore lab later demonstrated that gene rich regions correlate with more disrupted chromatin fibres. Although this relationship is not directly indicative of transcription, it suggests that the de-condensed chromatin state is permissive for transcription (Gilbert et al., 2004).

Active genes also maintain lower levels of linker histone H1 (Tazi and Bird, 1990) which is thought to influence folding of the 30nm fibre, and suggests that linker histones can be a repressor of gene transcription (Thomas, 1984). Loss of linker histones results in global changes in chromatin structure and mitotic defects (Fan et al., 2005; Maresca, Freedman and Heald, 2005). Detailed studies demonstrated that the effect of linker histones on transcription was gene specific, mediated by the occlusion of transcription factors (Laybourn and Kadonaga, 1991; Shen and Gorovsky, 1996; Crane-Robinson, 1999). While triple knockouts in mice are embryo lethal, individual knockouts do not effect growth suggesting that these gene specific H1 functions are not essential or that there is significant compensation mechanisms (Takami and Nakayama, 1997; Hashimoto et al., 2010).
The expression levels of genes are also inversely correlated with DNA methylation. Many highly expressed genes involved in cellular homeostasis contain un-methylated 5’ CpG islands (Mikkelsen et al., 2007). These ubiquitously transcribed promoters also exhibit decreased linker histone levels (Kamakaka and Thomas, 1990). Recent work demonstrated that promoters with CpG islands resulted in a nucleosome depleted region independent of transcription levels (Valouev et al., 2011; Fenouil et al., 2012). The functional role of CpG islands and these depleted regions is a current area of debate. It’s generally accepted that the nucleosome depletion increases accessibility to DNA binding transcription factors. Unpublished work from the Gilbert lab also suggests that these 5’ CpG islands have a structural function in facilitating transcription mediated supercoiling. Furthermore, the Balasubramanian lab has proposed the existence of the exotic DNA structure the G-quadruplex at promoters (Huppert and Balasubramanian, 2007) whilst further experiments have demonstrated that the stability of G-quadruplexes within promoters positively correlates with transcription (Renčiuk et al., 2017).
Recently RNA has been associated with chromatin de-condensation upon gene activation. As mentioned earlier, interspersed repetitive elements, Line-1 and CoT-1 transcripts function within the nuclear scaffold, maintain the euchromatic de-condensed state (Hall, Lisa et al., 2014). Complimentarily long non-coding RNA transcripts (lncRNA) from enhancers (eRNA) throughout the human genome have been implicated in gene activation (Andersson et al., 2014; Pefanis et al., 2015). These >200 bp transcripts are transcribed by RNA polymerase II (polII), lack open reading frames, and can be post-translationally modified (Kung, Colognori and Lee, 2013). However unlike other lncRNAs, eRNAs are rarely spliced and are rapidly degraded by the exosome. This results in most eRNA transcripts fractionating with chromatin bound proteins (de Santa et al., 2010). Recent studies have demonstrated that exosome mediated termination of PolII enables nascent eRNAs to recruit transcription factors to the local environment (Lemay et al., 2014; Sigova, 2015). Correspondingly, eRNAs are proposed to promote site specific chromatin disruptions to facilitate gene activation (Mousavi et al., 2013). Furthermore, seminal experiments from Nozawa et al., (2017) demonstrated that RNA in conjunction with SAF-A is required for transcriptional de-condensation of the chromatin fibre.
1.4.1 Facultative gene silencing

The basal chromatin state is broadly considered to be repressive. However, promoting this condensation further may be necessary to prevent aberrant transcription. As touched upon earlier, ncRNAs have been associated with classical X-inactivation, and recruitment of polycomb complexes (PcG). First identified in *Drosophila*, antipodal PcG and thrithorax complexes transiently modulate developmental gene expression in a tissue specific manner (Ingham, 1983). PcG proteins form PRC1 or PRC2 complexes depending on the presence of subunit RING1A/B or EZH1/2 respectively. PRC2 functions as a H3K27 histone methyltransferase (Kuzmichev *et al.*, 2002) but regulation of PRC2 targeting remains to be fully elucidated. Along with lncRNA HOTAIR, JARID2 and PHF19 have been implicated in directing PRC2 recruitment (Pasini *et al.*, 2010; Walker *et al.*, 2010). JARID2 functions with PRC2 component AEBP2 to facilitate *de novo* H3K27 methylation, although mechanistically this is not precisely defined (Son *et al.*, 2013). PRC2 subunit PHF19 via its tudor domain binds to the active histone mark H3K36me3 allowing repression of currently active genes in mESCs (Brien *et al.*, 2012; Cai *et al.*, 2013).

PRC1 core catalytic subunits, RING1A/B are E3 ubiquitinases targeting histone H2A lysine 119 (Wang *et al.*, 2004). Canonical targeting of PRC1 acts via subunits with CBX domains, which recognise the PRC2 modification H3K27me3 (Levine *et al.*, 2002; Fischle *et al.*, 2003). Non-canonical PRC1 complexes contain RYBP proteins instead of CBX proteins allowing PRC1 targeting independent of H3K27me3 (Tavares *et al.*, 2012; Gao *et al.*, 2013). Recent studies have now implicated non-canonical PRC1 complexes in
recruitment of PRC2 (Blackledge et al., 2014). The complex regulation PcG demonstrates the need for tight regulation of gene repression for correct development and disease prevention.

Polycomb dependent compaction was demonstrated cytologically by FISH (Eskeland et al., 2010), but this was independent of RING1B catalytic activity. Binding of PRC1 prevents transcription factor binding resulting in the inability to form a preinitiation complex (PIC) required for transcription (McCall and Bender, 1996; Lehmann et al., 2012). Polycomb binding also precludes SWI/SNF nucleosome remodelling maintaining a condensed repressive state (Shao et al., 1999). Maintaining this condensed state requires an evolutionarily conserved highly charged domain on PRC1 thought to function in its recruitment (Grau et al., 2011). Meticulous super resolution microscopy from Boettiger et al., (2016) demonstrated that PRC1 mediated large scale chromatin compaction occurs beyond transcriptional inactivation and across subdomains, showing it has a potentially larger role in genome organisation.

Polycomb mediated condensed chromatin fibres, as previously mentioned, are enriched for linker histones. Recent work has focused on the role of linker histone subtypes in maintaining this repressed chromatin structure. Unlike total linker histone, which is distributed genome-wide; linker histone variants form distinct topological domains. Linkers H1.2-H1.5 associate with HP1 domains at the nuclear periphery and are depleted from transcriptionally active regions. H1.1 by contrast is found at PcG domains, and weakly enriched at transcribed regions (Izzo et al., 2013). This suggests that linker histone H1.1 functions complementary to polycomb in transient gene repression.
1.5 Constitutive heterochromatin

Position-effect variegation (PEV) was first defined by changes in the colour pattern in *Drosophila* eyes (Muller, H, 1930). This change in colouration is caused by the translocation of an eye pigment gene to the heterochromatic regions surrounding the centromere. The propagation of heterochromatic chromatin structure across the pigment gene occurs heterogeneously correlating with the patchy pigment distribution across the eye. Molecular studies show this clear phenotype directly relates to transcriptional repression, and chromatin compaction, over the translocated ‘white’ pigment gene. This enabled genetic screening to identify factors that suppress or enhance the variegation of gene expression across the eye. Suppressors of variegation; Su(var)3-7, Su(var)2-5 (also named HP1), and Suv(var)3-9 all demonstrated a dose dependent effect on PEV demonstrating their key function in heterochromatin propagation (Reuter *et al.*, 1990; Eissenberg *et al.*, 1992; Tschiersch *et al.*, 1994). Additionally translocation screens identified multiple other loci across the chromosome that exhibited position-effect variegation including telomeres (Doheny, Mottus and Grigliatti, 2008; Riddle *et al.*, 2008). The heritable state of surrounding chromatin has since been demonstrated across multiple eukaryotes (Ottaviani, Gilson and Magdinier, 2008). However, as PEV sites are defined by the underlying chromatin structure, they were demonstrated to diverge between cell types (Hofmann, Brünner and Korge, 2009). This body of work was the foundation of our current understanding that chromatin structure is a major mechanisms for gene activation and repression.
The relationship between chromatin structure, nuclear organisation and transcription has led to much debate as to the causative agent behind genome regulation. This has been muddied by correlations with histone modifications and replication timing. Heterochromatin is strongly associated with the nuclear periphery and the idea of nuclear organisation associating with chromatin regulation was first put forward by Carl Rabl, (1885) observing peripheral centromere clustering in salamander nuclei.

A causative function of nuclear organisation was first put forward in *drosophila* (Dernburg et al., 1996). Heterochromatic repeats were inserted near to the brown eye colour gene and using FISH they demonstrated that its nuclear distribution was not altered in embryonic cells. However, upon reaching the larval stage, the brown gene is tethered with other heterochromatic regions to the periphery supressing its expression (Dernburg et al., 1996). Mammalian studies in primary B-lymphocytes have also shown inactive genes localising to the periphery in cycling but not quiescent cells. In these phenotypically similar cells without nuclear peripheral localisation, the heterochromatin foci were not heritable suggesting that nuclear organisation is required to maintain chromatin structure and transcriptional profiles (Brown et al., 1999).
More recent studies furthered this work by showing that the early B cell factor (EBF1) locus relocates from the periphery to a more central position at the same stage of differentiation as its expression (Lin et al., 2012). This effect can occur at single genes or at regions with multiple genes (Peric-hupkes et al., 2010). An eloquent study recently showed that forcing a loop between the β-globin locus and its long-range control region without canonical transcription factor GATA-1 was sufficient to induce transcription (Deng et al., 2012). Additionally, forcing this loop to γ-globin resulted in its up regulation at the expense of β-globin, demonstrating a clear link between genome organisation and transcription (Deng et al., 2014).

Adversely, the Bickmore lab recently demonstrated that de-condensation of peripheral chromatin without transcription was enough to drive gene relocation away from the nuclear periphery. This did not, however, affect replication timing unless transcription was induced (Therizols et al., 2014). Lack of coupling between nuclear organisation and replication, however conflicts with other studies demonstrating TAD establishment in early G1 correlated with replication timing determination (Dileep et al., 2015). The Misteli lab identifying multiple replication components affecting nuclear positioning supported this theory (Shachar et al., 2015). They also demonstrated knockdown of chromatin structure and SWI/SNF remodelling components caused highly expressed genes to be relocated to the periphery while maintaining high expression levels (Shachar et al., 2015). Thus the causative nature of silencing at the nuclear periphery is not universal, and remains poorly understood.
Heterochromatic histone modifications are also proposed to regulate the chromatin fibre. Heterochromatin is acetylated in yeast and *Drosophila* at H4K12 (Turner, Birley and Lavender, 1992; Braunstein *et al.*, 1996). However, acetylation in humans is excluded from the heterochromatic inactive X chromosome (Jeppesen and Turner, 1993). This hypoacetylated state, was demonstrated to regulate chromatin compaction and gene silencing by the Belmont lab. Targeting a VP16-lac repressor to a genomic region with an array of lac operator sequences, caused localised hyperacetylation, transcription, and chromatin unfolding (Tumbar, Sudlow and Belmont, 1999). Correspondingly, artificial recruitment of suppressor of variegation 3-9 homologue 1 (Suv39h1) or polycomb protein EZH2 was sufficient to reposition subTAD domains to polycomb bodies or pericentric heterochromatin respectively. With the recruitment of Suv39h1 repositioning occurred from an active and inactive domain. However Suv39h1 mediated repositioning requires the chromodomain not the methyltransferase activity of the enzyme and appears independent of transcriptional state (Wijchers *et al.*, 2016).

Intriguingly, dense heterochromatin is not continually refractory to gene transcription and contains multiple unique gene transcripts in *Drosophila* (Coulthard *et al.*, 2010). The requirement of constitutive heterochromatic domains for correct transcription of these genes was eloquently demonstrated by translocation studies from the Hilliker lab (Eberl, Daniel, Duyf, Brenda and Hilliker, Arthur, 1993). Key regulators of PEV, Su(var)3-7 and HP1, have subsequently been linked to correct expression of heterochromatic genes and piRNAs respectively (Lu *et al.*, 2000; Basquin *et al.*, 2014).
The interplay between nuclear positioning, chromatin structure, histone modifications, and replication has been extensively studied in relation to gene regulation. The complexity of this system is demonstrated by the amount of conflicting studies in different systems. Pioneering work by Larson et al., (2017) recently demonstrated that the formation of heterochromatin via suppressor HP1α occurs in phase separated droplets. Loss of this phase separation environment results in a decrease in HP1α foci size and frequency in cells. This suggests that the liquid environment around chromatin may itself contribute to chromatin structure regulation, demonstrating the unknown complexities that have yet to be fully elucidated.

Figure 1.4 Subdiffraction resolution imaging with SIM. (A) Cross section of mouse muscle myoblast C2C12 cell nucleus stained with DAPI and imaged by conventional epifluorescence microscopy. (D) The same cross-section imaged by 3D SIM reconstruction. Bright aggregates are constitutive heterochromatic chromocenters. Increase in DAPI intensity at the nuclear periphery is indicative of the heterochromatinisation of the adjacent chromatin. Scale bars are 5 µm. Adapted from Schermelleh et al., (2008)
1.5.1 Centromeres

The principal cytological constriction of human metaphase chromosomes, the centromere, was first observed by Walther Flemming (1882). However, due to stark heterogeneity across species, Cyril Darlington (1936) argued a centromere must be defined by its function. Centromeres function as loading platforms for spindle microtubules, in order to control chromosome movement throughout mitosis. Although most eukaryotes contain a monocentric restriction, some species have diffuse centromeres along whole chromosomes termed holocentricity (Guerra et al., 2010).

The location of monocentric centromeres is characterized by the deposition of histone variant CENP-A or its homologue, in most eukaryotes (Earnshaw and Rothfield, 1985). Artificial targeting of CENP-A is sufficient for kinetochore assembly, indicating its role to nucleate centromere formation (Mendiburoa et al., 2011). This allows centromere location to be independent of underlying DNA sequences and can heterogeneously drift between cells (Yao et al., 2013; Purgato et al., 2015). The amount of CENP-A required for centromere formation is also variable, with a single CENP-A required in some budding yeasts to kilobase regions of CENP-A deposition (Pluta et al., 1995; Furuyama and Biggins, 2007; Bodor et al., 2014). Regional centromeres are composed of a central core where CENP-A is deposited, and an outer heterochromatic domain defined as the pericentromere.
The underlying centromeric DNA sequences also demonstrate evolutionary variability, thought to be a by-product of meiotic drive in female meiosis (Malik and Henikoff, 2009). Most centromeres form over complex arrays of highly reiterative DNA sequences. These repetitive arrays are AT-rich domains interspersed with retrotransposon clusters, while the central core typically has homogenous ordered repeats, the pericentromere is less ordered and often has multiple repeat sequences (Fishel et al., 1988; Schueler et al., 2001).

These repetitive elements have increased frequency of meiotic recombination due to their sequence similarity. This may decrease genome stability when their chromatin environment is accessible, and therefore requires a more compact chromatin state (Vader et al., 2011), alternatively a regular chromatin structure may be required to form a stable kinetochore. These compact chromatin fibres were first described as satellite bands isolated from bulk DNA in density gradients (John and Miklos, 1979). One of the most abundant, the α-satellite, is conserved across primates spanning 5% of the genome. α-satellite in primates comprises a core 171 bp repeat that hybridises to all human canonical centromeres (Fittler and Zachau, 1979). Within the central core of the centromere, sixteen α-satellite monomers are arranged sequentially head to tail forming a 2.7 kb higher order repeat unit propagated throughout. By contrast, the human pericentromere contains disordered adjacent monomers (Aldrup-MacDonald and Sullivan, 2014). Nucleosome positioning is strictly defined across α-satellites, first demonstrated in African green monkeys (Zhang, Fittler and Horz, 1983). Mice by contrast have only two centromeric repeats, major and minor satellites. The centric minor satellite has a shorter 120 bp repeat and the major pericentric repeat is 234 bp (Wong and Rattner, 1988).
Figure 1.5 Centromere structure and organisation. (A) Typical acrocentric mouse chromosome, with pericentric major satellite and centric minor satellite (red) repeats. (B) Centromere identity components. The CENP-A core contains H3K36me, H3K4me2, and acetylation, whereas pericentric regions contain H3K9me3. (C) Major satellite organisation (red) in mouse 3T3 cells mitosis or interphase by DNA fluorescence in situ hybridisation (FISH). Scale is 10 µm. Adapted from Probst and Almouzni, (2011); Lyn Chan and Wong, (2012)
\textbf{1.5.2 Centromere large-scale structure}

The role of CENP-A in large scale chromatin fibre structure at centromeres, is limited by its low frequency of deposition (~400) within the core centromeric region contributing to just ~4\% of nucleosomes (Bodor et al., 2014). High-resolution microscopy of stretched chromatin fibres demonstrated that these were ordered into blocks of CENP-A or H3 containing nucleosomes from \textit{Drosophila} to humans (Blower, Sullivan and Karpen, 2002; Ribeiro et al., 2010). Electron microscopy experiments demonstrated that CENP-A nucleosomes are exclusively found at the surface of the cytological constriction. The CENP-A nucleosomes are not prevalent enough though to cover the entire surface of the restriction, occupying two thirds of the length, and one third of both the width and height of the surface (Marshall, Marshall and Choo, 2008). How the chromatin fibre is compacted to allow these CENP-A domains to lie on the surface of the fibre remains to be elucidated. Many models including; looping, solenoidal, planar sinusoids, and layered boustrophedon have been hypothesised. The repetitive nature of satellite sequences makes the use of capture-C methods problematic (Naumova et al., 2013). Additionally, limitations of current microscopy techniques mean that the folding model of the core centromere remains an area of current debate.
Flanking the core centromere, the pericentromere contains canonical heterochromatic marks and is responsible for sister chromosome cohesion with high levels of cohesion (Glynn et al., 2004). This process is mediated by the stabilisation of cohesion complexes throughout S phase (Yeh et al., 2008). The heterochromatic nature of the pericentric regions directly mediates this process via H3K9me3. In *S. pombe*, HP1 homologues Swi6 and Chp2 which bind H3K9me3 provides a platform for cohesin recruitment (Bernard et al., 2001; Nonaka et al., 2002). Pericentric cohesin is protected from turnover by Swi6 recruiting shugoshin proteins (Yamagishi et al., 2008). These recruit phosphatase PP2A preventing cohesin subunit phosphorylation and subsequent separase cleavage (Kitajima et al., 2006). The role of H3K9me3 and HP1 in regulating mammalian cohesin remains controversial. While HP1 in humans recruits shugoshin proteins, they are not essential for cohesin protection and do not drive cohesin recruitment (Koch et al., 2008; Serrano, Rodríguez-Corsino and Losada, 2009). However, Suv4-20 methyltransferase, tethered by HP1 isoforms significantly contributes to loading cohesin (Yang et al., 2008; Shimura et al., 2011; Hahn et al., 2013). Loss of pericentric cohesin loading completely abrogates the cytological constriction known as heterochromatin repulsion (Whelan et al., 2012). This occurs in the human disease Roberts syndrome, causing increased aneuploidy and abnormal nuclear morphology due to loss of genome integrity during mitosis (Jabs et al., 1991). Despite the function of heterochromatin and cohesins during mitosis, the loss of Suv39 methyltransferases in mice does not lead to loss of their cytological constriction and remarkably are viable with few minor abnormalities (Peters et al., 2001).
1.5.3 Neocentromeres

Despite the highly ordered nature of repetitive elements within the centromere, they are not essential. Centromeres can form sporadically over non-repetitive DNA, classed as neocentromeres. These were first found to occur naturally in humans by Voullaire et al., (1993) and can be stably passed generation to generation (Tyler-Smith et al., 1999). In Drosophila irradiation exposure was sufficient to produce acentric mini-chromosomes containing a neocentromere (Murphy and Karpen, 1995). These mini-chromosomes demonstrated the ability to recruit centromere specific protein ZW10, and formed a neocentromere from the sub-telomeric region of chromosome X (Williams et al., 1998). This sub-telomeric sequence does not display the cytological restriction of a centromere when not attached to the X chromosome outside of this mini-chromosome environment, further demonstrating the epigenetic activation step required for centromere formation.

Dicentric chromosomes occur when a centromere naturally translocates within a chromosome resulting in inactivation of the original centromere locus. The dicentric (X;15) translocation in Drosophila first demonstrated the loss of centromeric proteins such as CENP-C from the inactive centromere (Page et al., 1995). Additionally heterochromatic proteins HP1β and Suv39h1 are found pericentrically at all canonically active but not inactive centromeres (Aagaard et al., 2000; Saffery et al., 2000). The loss of these heterochromatic proteins elevates chromosome loss suggesting impaired kinetochore function (Taddei et al., 1999; Rea et al., 2000). Furthermore, inner centromere protein (INCENP) directly associates with HP1 (Ainsztein et al., 1998) and binds to the spindle mid-zone as part of the chromosome
passenger complex for chromosome arrangement throughout mitosis (Mackay et al., 1998). Despite INCENP’s direct interaction with HP1, Ainsztein demonstrated this was not required for centromere targeting of INCENP, instead functioning in its transfer from chromosomes to the anaphase spindle. Recently the role of pericentric heterochromatin has come under debate as neocentromeres can occur without surrounding heterochromatic marks H3K9me3 and HP1, albeit with centromere cohesion defects (Alonso et al., 2010). This demonstrates a functional but not essential role of heterochromatin for centromere formation.

1.5.4 Evolutionary New Centromeres

The ability to form non-repetitive centromeres appears across all forms of life. Some species, such as orangutan, horse and chicken, have both repetitive and non-repetitive centromeres within their genomes (Piras et al., 2010; Shang et al., 2010; Locke et al., 2011). By comparing recently diverged evolutionary species these centromeres have been termed evolutionary new centromeres (ENCs) (Kasai et al., 2003; Rocchi et al., 2012). This centromere repositioning occurs without transposition of the surrounding genetic landscape (Montefalcone et al., 1999). Intriguingly however a typical ENC acquires the molecular properties and satellite repeat sequences of ‘old’ centromeres. Macaques, in contrast to orangutans, have nine ENCs within their genome, each of which contains α-satellite duplications (Ventura et al., 2007). Therefore, despite not being required for centromere formation, there is evolutionary pressure for their presence. Furthering this idea, the satellite arrays at old centromere positions decay once ENCs are formed (Kalitsis and Choo, 2012).
1.5.5 Mammalian Artificial Chromosomes

In order for a chromatin fibre to be a stable constituent of a genome, it requires the ability to undergo mitosis. In order to be correctly segregated in mitosis, a functioning centromere is required. Generation of *S. cerevisae* artificial chromosomes (YACs) was therefore relatively simple as the single CENP-A requires a specific underlying DNA sequence (Clarke, 1998). Human artificial chromosome production proved more intricate as the underlying factors of centromere formation remain unresolved. Pioneering work by Harington *et al.*, (1997) combined \( \alpha \)-satellite arrays with telomeric and genomic DNA in human HT1080 cells to generate a 6-10Mb chromosome, further demonstrating the role of repetitive higher order units in centromere structure. Although the mechanism behind artificial centromere formation is yet to be fully elucidated, recent studies propose that \( \alpha \)-satellite has a propensity for epigenetic marks that favour CENP-A deposition (Bergmann *et al.*, 2011; Ohzeki *et al.*, 2012).
1.5.6 Regional Centromere Determinants

For genomic sequences such as $\alpha$-satellite to be a determining factor in centromere location, they must directly or indirectly recruit CENP-A and the constitutive centromere associated network (CCAN). In *S. cerevisiae* the single CENP-A nucleosome location is determined by the presence of the centromere DNA element III (CDEIII) recognised by Cbf3 (Lechner and Carbon, 1991).

Regional centromere sequences across species are conversely highly varied, unlike the components of CCAN. CCAN component CENP-C for instance has high homology with *S. cerevisiae* Mif2 protein (Meluh and Koshland, 1995). Ectopic recruitment of core CCAN components CENP-C, HJURP, and CENP-I individually is sufficient for CENP-A deposition and kinetochore assembly at centromeres (Hori *et al.*, 2013). This demonstrates that the targeting of core CCAN is the main determinant for centromere location. In contrast to the conservation of CCAN, the only conserved centromeric sequence from humans to mice is the 17 bp CENP-B box sequence (Muro *et al.*, 1992). The CENP-B box is present in the higher order repeats of the core minor satellite region in *M. musculus* and human $\alpha$-satellite. CENP-B, which binds to this sequence, directly stabilizes CENP-A nucleosomes and their interaction with CCAN component CENP-C (Fachinetti *et al.*, 2015). While CENP-B increases the fidelity of centromere location, loss of the CENP-B box in mice is not lethal (Hudson *et al.*, 1998) Additionally other primate and rodent species have divergent sequences or lack the sequence entirely (Haaf *et al.*, 1995; Kipling *et al.*, 1995). As expected, CENP-B is not recruited to neocentromeres, and intriguingly Y chromosome centromeres lack the CENP-B box repetitive sequence and subsequent CENP-B binding.
Epcle of repetitive elements from the Y chromosome however are not able to form HACs which require the CENP-B box (Grimes, Rhoades and Willard, 2002; Ohzeki et al., 2002). This further demonstrates a functional if not essential role for the CENP-B box centromeric sequence.

In addition to the underlying genomic sequence at centromeres, specific epigenetic states are thought to be permissive to centromere formation. H3 variant CENP-A as mentioned is required for recruitment of kinetochoore components. The effect of CENP-A deposition on a nucleosome’s physical property at the centromere remains unclear. Whether CENP-A is found in an octamer or a tetramer hemisome in vivo is the crux of this debate (Dunleavy, Zhang and Karpen, 2013). Perceptive hydrogen-deuterium exchange experiments demonstrated that CENP-A--H4 tetramers are more compact due to a rotated interface between the two CENP-A molecules (Sekulic et al., 2010). Additional studies have demonstrated the ability of CENP-A hemisomes to preferentially wrap DNA in a right handed manner (Furuyama and Henikoff, 2009). Contrary to this, crystal structures of octameric CENP-A nucleosomes do not perform this axis rotation and form more condensed arrays (Panchenko et al., 2011; Tachiwana et al., 2011). These views were unified recently by Falk et al., (2015), who demonstrated that both forms are present in solution with the octameric form preferring CENP-C binding.

The local chromatin environment is proposed to regulate histone variant CENP-A deposition throughout the core centromere. Unlike normal nucleosome incorporation, CENP-A is uncoupled from replication and remains in a hemi state throughout mitosis (Shelby, Monier and Sullivan,
Histone variant H3.3 incorporates where CENP-A is lost acting as a marker for new assembly of CENP-A nucleosomes in G1 (Dunleavy, Almouzni and Karpen, 2011). Incorporation of CENP-A is facilitated through chaperone complexes containing HJURP and MIS18 proteins. While not all MIS18 components are evolutionarily conserved, MIS18 complexes have been shown to interact with CENP-C across species (Moree et al., 2011; Dambacher et al., 2012). CENP-C has also been demonstrated to directly interact with CENP-A chaperone HJURP demonstrating its ability facilitate CENP-A—CENP-C interactions (Tachiwana et al., 2015).

In addition to CENP-C and H3.3 presence at centromeres, surrounding chromatin signatures influence CENP-A deposition. For instance MIS16 and MIS18 have been proposed to prime the centromere for CENP-A deposition via DNA methylation and histone deacetylation (Hayashi et al., 2004; Kim et al., 2012). Pericentric regions as mentioned above canonically contain heterochromatic marks such as H3K9me3 and DNA methylation (Lehnertz et al., 2003). Adversely, the CENP-A core region contains transcriptionally permissive marks, H3K4me2 and H3K36me2 (Sullivan and Karpen, 2004). This chromatin state has been demonstrated to directly influence CENP-A deposition by HJURP (Bergmann et al., 2011). Eloquent studies on human artificial chromosomes demonstrated that the heterochromatinization of the inner centromere core abdicated centromere function and CENP-A deposition (Nakano et al., 2008; Ohzeki et al., 2012).

The requirement of this permissive chromatin state at centromeres has led to a hypothesis for transcription at the core centromere. Transcription machinery including FACT (facilitates chromatin transcription complexes),
RSF1, and helicase CHD1 have all been implicated in CENP-A deposition (Perpelescu et al., 2009; Dudek et al., 2010). Absence of FACT mediated transcription results in aberrant CENP-A deposition and centromere function, demonstrating a direct link between transcription and centromere function (Chen et al., 2015).

The presence of RNA transcripts from these repetitive elements appears to be required. In *S. pombe* the RNA interference complex DICER cleaves pericentric transcripts, producing small interfering RNA (siRNA). These short transcripts and HP1 are required for correct CENP-A deposition (Folco et al., 2008). The function of transcription at human centromeres has also been inferred by the presence of polymerase II and transcription during mitosis, while the Gilbert lab (unpublished) shows transcription during interphase at human neocentromeres (Chan et al., 2012). Investigations using HACs has also shown the presence of α-satellite transcripts from its centromere (Bergmann et al., 2011). Disrupting this transcription at the centromere results in kinetochore defects and aberrant CENP-A profiles (Quénet and Dalal, 2014; Liu et al., 2015).

Together this body of work demonstrates that interplay of genomic sequences, local chromatin states, transcription and CCAN formation occurs. Exactly how these contribute to the spatial restriction of the centromere and its propagation remains elusive. This remains an area of current major scientific interest as the field looks to understand why some abnormal CENP-A deposition induces neocentromere formation, while other sites remain inert. Understanding this process will provide intriguing evolutionary insights in addition to understanding the relationship between epigenetic states and chromatin fibre structure.
1.5.7 Telomeres

Telomeres are heterochromatic regions that cap linear chromosomes. They were first identified as a distinctive feature of nuclear structure in *drosophila* by Herman Muller, who noted their requirement for faithful genome maintenance (Muller, H, 1938). These chromosome caps function to distinguish the chromosome ends from breaks. This allows cells to undergo mitosis without activating cell cycle checkpoint responses to chromosome breaks (Sandell and Zakian, 1993). The requirement of telomeres for cell cycle progression has promoted their evolutionary conservation (Meyne, Ratliff and Moyzis, 1989).

Vertebrates have large kb arrays of repeating telomeric associated sequences (TAS) to cap their chromosomes. Although mostly double stranded, the definitive end is a 3’ single stranded overhang ranging from 50-400 nucleotides (Wright *et al.*, 1997). These exposed nucleotides are proposed to form a high order loop like structure, folding into the duplex region of the telomere, protecting chromosome ends from DNA repair mechanisms (Griffith *et al.*, 1999).
Loss or shortening of these capping sequences is associated with senescence, faithful chromatid separation, genome stability and oncogenesis (Lundblad and Blackburn, 1993; Greider, 1998; Blackburn, 2001). These phenotypes occur as DNA replication is not conservative and, therefore, completion to the chromosome end is not essential. Organisms must harbour mechanisms to restore lost telomeric sequences after every mitotic division to prevent loss of telomeric repeats. This is achieved by telomerase which uses RNA telomeric transcripts as a template for DNA restoration (Collins and Miller, 2002).

The stabilisation of the T loop structure and the accessibility of telomerase mediated repair is regulated by telosome or shelterin complexes (De Lange, 2005). Subunits TRF1/2 negatively regulate telomerase activity and in combination with POT1 facilitate T-loop formation (Stansel, De Lange and Griffith, 2001; Hockemeyer et al., 2006). Although telomere structures are required to avoid activating the DNA damage response pathway, shelterin recruits many DNA damage proteins involved in signalling, nucleotide excision, helicases, homologous repair (HR) and non-homologous end joining (NHEJ). These interactions all have a functional role in telomere maintenance. Loss of ATM-TRF2 interaction in ataxia-telangiectasia patients, for instance, results in aberrant telomere length (Hande et al., 2001; Karlseder et al., 2004). This is mediated by excision of the 3’ overhang followed by HR or NHEJ that also prevent chromosome end to end fusions (Zhu et al., 2003; Myung et al., 2004; Tarsounas et al., 2004).
Werner and Bloom syndrome patients with helicase mutations first implicated the role of chromatin structure on telomere maintenance. WRN and BLM helicases have since been shown to interact with TRF2 causing the de-condensation of telomeric regions (Opresko et al., 2002).

Mammalian chromatin condensation at telomeres was demonstrated by reduced nuclease accessibility in rats (Makarov et al., 1993). Subsequent studies have demonstrated enrichment for: constitutive heterochromatic HMT’s Suv39h1/2 and Suv420h1/2, their methylation marks H3K9me3 and H4K20me3, DNA methylation and HP1 (García-Cao et al., 2003; Gonzalo et al., 2006; Benetti et al., 2007). The formation of constitutive heterochromatin reduces the accessibility of telomerase enzymes, therefore, regulating telomere length. This is demonstrated by loss of Suv39h1/2 or DNMT’s causing telomere elongation, and HP1 overexpression instigating telomere erosion (García-Cao et al., 2003; Sharma et al., 2003; Gonzalo et al., 2006). Similarly to centromeric regions, transcription of telomeric repetitive elements has recently been implicated in its regulation. Telomeric RNAs (telRNA) are transcribed by PolII from the 5’ strand and therefore does not occur at the 3’ overhang. telRNA’s remain chromatin bound at telomeric regions by interacting with TRF1 and demonstrate the ability to inhibit telomerase activity (Schoeftner and Blasco, 2008). The role of these telRNA’s on chromatin structure has yet to be properly investigated, but provides another function for ncRNA in regulating heterochromatin structure.
1.6 Thesis Aims

The advancement of sequencing technologies has resulted in a more refined understanding of genome organisation and the association of proteins and their modifications. However, these studies have largely focused on euchromatin due to the repetitive nature of centromeric regions. The relationship between histone modifications and global chromatin structure is based on correlative rather than causative evidence, and a direct role continues to be debated. As such, whether both compaction and decompaction are active processes also remains unresolved. Identifying factors that affect heterochromatin formation, consequently, remains an important research question today.

Additionally, epigenetic regulation has been suggested as important therapeutic cancer targets (Feinberg, Ohlsson and Henikoff, 2006). However, drug development has largely focused on inhibiting oncogenes, rather than the re-expression of tumour suppressors, due to clarified mechanisms of action, and reduced potential off-target effects. Heterochromatic abnormalities and mutations in repressive epigenetic factors are however associated in disease phenotypes, suggesting their therapeutic potential (Ehrlich et al., 2002; Suzuki et al., 2004; Birgisdottir et al., 2006).

Thus this thesis aims to:

- Investigate any causality behind epigenetic regulation of chromatin structure correlations.
- Identify components of heterochromatin regulation
- Investigate the epigenetic regulation of acquired kinase signalling inhibitor resistance.
Chapter 2: Methods

2.1 General reagents, stock solutions and buffers

2.1.1 Sources of reagents

All solutions were prepared with deionised water and stored at room temperature unless stated otherwise.

Chemicals were purchased from Sigma Aldrich, Fischer Chemicals, GE healthcare, and VWR. Enzymes and antibodies were procured from New England Biolabs, Roche, Life Technologies, and Promega. Invitrogen manufactured all cell culture materials unless stated differently.
2.1.2 Stock solutions and buffers

20 × TBE 1L: 108 g Tris-base, 27.5 g boric acid, 40 ml 0.5 M EDTA

20 × TPE 1L: 218 g Tris-base, 31 ml orthophosphoric acid (85%), 40 ml 0.5 M EDTA

20 × TEEP 0.2L: 40 ml 1 M Tris (pH 8), 8 ml 0.5 M EDTA, 40 ml 100 mM EGTA, 0.05% (w/v) NP40, 250 μ M PMSF

5 × DNA loading buffer: 50% glycerol, 5 mM EDTA pH 8, 0.3% orange G (w/v)

4 × SDS buffer: 40% glycerol v/v, 8% SDS v/v, 0.25 M Tris-HCl pH 6.8, 5 mM β-mercaptoethanol

Buffer 1: 0.5 M Tris pH 8, 0.15 M NaCl

Cell lysis buffer: 85 mM KCl, 0.5% NP-40, 5 mM HEPES

ChIP dilution buffer: 0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl pH 8, 167 mM NaCl.
<table>
<thead>
<tr>
<th><strong>Chloroform-IAA:</strong></th>
<th>Chloroform and iso-amyl alcohol were mixed at a 24:1 ratio</th>
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<tbody>
<tr>
<td><strong>Colloidal Coumassie Stain:</strong></td>
<td>0.25% (w/v) brilliant blue R-250, 45% methanol, and 10% glacial acetic acid.</td>
</tr>
<tr>
<td><strong>Colloidal Coumassie Destain:</strong></td>
<td>30% methanol, 10% glacial acetic acid</td>
</tr>
<tr>
<td><strong>Chromatin elution buffer:</strong></td>
<td>1% SDS, 0.1 M NaHCO₃</td>
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<tr>
<td><strong>CSK buffer:</strong></td>
<td>100 mM NaCl, 0.1% Triton X-100, 300 mM sucrose, 1 mM MgCl₂, 1 mM EGTA, 10 mM PIPES and 100 μM PMSF.</td>
</tr>
<tr>
<td><strong>Destaining solution:</strong></td>
<td>30% methanol, 7% glacial acetic acid, 63% ddH₂O (v/v)</td>
</tr>
<tr>
<td><strong>Denaturation buffer:</strong></td>
<td>1.5 M NaCl, 0.5 M NaOH</td>
</tr>
<tr>
<td><strong>Genomic lysis buffer:</strong></td>
<td>150 mM NaCl, 1% (w/v) SDS, and 10 mM EDTA</td>
</tr>
<tr>
<td><strong>Hybridisation buffer:</strong></td>
<td>6 × SSC, 10 mM EDTA, 5 × Denhardt’s solution, 0.5% SDS, 100 μg/ml salmon sperm DNA.</td>
</tr>
</tbody>
</table>
**In vitro** click reaction mix:

3 µl 2 M triethylammonium acetate buffer, 30 µl DMSO, 6 µl 5 mM ascorbic acid, 3 µl 10 M CuTBTA, 1.2 µl 10 mM biotin azide

LiCl buffer

0.25 M LiCl, 1% NP-40, 1% deoxycholate, 1 mM EDTA, 10 mM Tris-HCl pH 8.

Luria-Bertani (LB) agar (1L):

10 g tryptone, 5 g yeast extract, 10 g NaCl, 15 g agar, pH 7.0 by sodium hydroxide. Prepared by the MRC HGU Technical Services.

Luria-Bertani (LB) broth (1L):

10 g tryptone, 5 g yeast extract, 10 g NaCl, pH 7.0 by sodium hydroxide. Prepared by the MRC HGU Technical Services.

MD Anderson lysis buffer:

1% Triton X-100, 50 mM HEPES, 150 mM NaCl, 1.5 mM MgCl₂, 1 mM EGTA, 199 mM NaF, 10mM sodium pyrophosphate, 1 mM Na₃VO₄, 10% glycerol, phosphatase inhibitor cocktail (Roche, cat no. 04 906 845 001)
Nuclear Buffer A (NB A): 85 mM KCl, 5.5% sucrose, 10 mM Tris pH 7.6, 0.5 mM spermidine, 0.2 mM EDTA, 250 μM PMSF

Nuclear Buffer B (NB.B): Nuclear buffer A supplemented with 0.2% NP-40 for differentiated cells, and 0.05% NP-40 for ESCs

Nuclear Buffer Release 2: 85 mM KCl, 5.5% sucrose, 10 mM Tris-HCl pH 7.6, 1 mM CaCl2, 1 mM MgCl2, 250 μM PMSF.

Nupage transfer solution: 1 × Nupage transfer buffer, 10 or 20% methanol, 1% nupage antioxidant

Neutralisation buffer: 1.5 M NaCl, 0.5 M Tris-HCl pH 7.5

Optimal live cell click reaction: 0.5 mM CuSO4, 0.25 mM THPTA, 250 mM sodium ascorbate

Phosphate buffer saline (PBS): DuBlecco’s recipe (without calcium and magnesium), 10 mM Phosphate, 137 mM NaCl, 27 mM KCl. Prepared by MRC HGU Technical Services

Penicillin/Streptomycin: 10,000 U/ml penicillin and 650 μg/ml streptomycin. Prepared by MRC HGU Technical Services
<table>
<thead>
<tr>
<th>Component</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Propidium iodide solution</td>
<td>PBS, 1 μg/ml propidium iodide, 4 μg/ml RNase A.</td>
</tr>
<tr>
<td>Proteinase K</td>
<td>20 mg/ml (w/v) proteinase K, 1.5 mM calcium acetate, 50% glycerol, 50 mM Tris-HCl pH 7.</td>
</tr>
<tr>
<td>RIPA</td>
<td>1% NP-40, 0.5% sodium deoxycholate (v/v), 0.1% SDS in water. Protein inhibitor cocktail added before use.</td>
</tr>
<tr>
<td>Saline sodium citrate (SSC)</td>
<td>0.5L 20 × stock prepared by MRC HGU Technical Services: 3 M NaCl, 0.3 M trisodium citrate, pH 7.</td>
</tr>
<tr>
<td>TEEP Δ NaCl</td>
<td>20 × TEEP stock used to make 10 mM Tris-HCl pH 7.5, 1 mM EDTA, 1 mM EGTA, 250 μM PMSF, supplemented with 1 mM, 20 mM or 80 mM NaCl.</td>
</tr>
<tr>
<td>TBE</td>
<td>90 mM Tris-borate, 2 mM EDTA prepared from 20 × stock</td>
</tr>
<tr>
<td>TBS-T</td>
<td>50 mM Tris-HCl, 150 mM NaCl, 0.05% Tween-20</td>
</tr>
<tr>
<td>Buffer Type</td>
<td>Composition</td>
</tr>
<tr>
<td>---------------------</td>
<td>-----------------------------------------------------------------------------</td>
</tr>
<tr>
<td>Tris EDTA (TE)</td>
<td>10 mM Tris-HCl, 0.1 mM EDTA. 50 ml stock was prepared by MRC HGU Technical Services.</td>
</tr>
<tr>
<td>TPE</td>
<td>0.8 M Tris-phosphate, 0.02 M EDTA prepared from 20 × stock.</td>
</tr>
<tr>
<td>TSE-I buffer</td>
<td>0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl pH 8, 150 mM NaCl</td>
</tr>
<tr>
<td>TSE-II buffer</td>
<td>0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl pH 8, 500 mM NaCl</td>
</tr>
</tbody>
</table>
2.2 Bacterial Culture

2.2.1 Media

*E. coli* strains were grown at 37°C in a Luria-Bertani (LB) broth suspension by a shaking incubator (250 rpm) overnight. To select for plasmid containing bacteria, ampicillin or kanamycin was added as required. Ampicillin resistant *E. coli* colonies were grown on ampicillin containing L-agar plates poured by MRC HGU Technical Services.

2.2.2 Bacterial strains

DH5α competent cells (Invitrogen) were used for cloning experiments, and stored at -80°C.

2.2.3 Bacterial glycerol stocks

50% glycerol stocks were made for long-term bacterial storage. 500 μl of *E. coli* suspended in LB broth was added to 500 μl of glycerol and stored at -80°C.

2.2.4 Bacterial transformation

50 μl of DH5α competent cells (Invitrogen) and thawed on ice for 15 minutes then 50 ng of plasmid or ligation was added. Competent cells were heat-shocked at 42 °C for 30 seconds before being rested on ice for 2 minutes to reduce the temperature. 400 μl of SOC media was then added to the bacterial culture and incubated at 37 °C for 30 minutes. Cultures were diluted and spread on L-agar plates with antibiotics, as required.
2.3 DNA methods

2.3.1 Phenol/chloroform DNA purification

To purify mammalian genomic DNA, cells were trypsinised and suspended in genomic lysis buffer (GLB). RNase A/T1 mix (Cat No EN0551) was added to the GLB lysed solution to a final concentration of 5 μg/ml of RNase A and 12.5 U/ml of RNase T1. Samples were incubated at 37 °C for 30 minutes. Proteinase K at 150 μg/ml was added to the solution and further incubated at 55 °C for 2-16 hours. Phenol chloroform-IAA at a ratio of 25:24:1 was added in equal volume to the solution before inversion to mix. Samples were centrifuged at 12,000 g for 30 minutes at room temperature to separate the different phases. The aqueous phase was transferred to a new tube and re-extracted with chloroform-IAA, in order to remove residual phenol.

DNA was precipitated from samples by the addition of sodium acetate (pH 5.5) to 300 mM with 2.5 volumes of ethanol. 1 μl of glycogen 20 μg/ml (Sigma cat no G1508) was added as a carrier and incubated at -20°C for 1-16 hours. The DNA was collected by centrifugation at 12,000 g for 30 minutes at RT. The supernatant was removed and the pellet was washed with 1ml 70% ethanol to remove residual salt. The DNA was re-centrifuged at 12,000 g 10 minutes at RT. The supernatant was again removed and the pellet dried at room temperature. DNA was resuspended in TE and quantified by 260 nm absorbance on a Nanodrop 1000 UV-VIS spectrometer (Thermo Scientific). The 260/280 ratio was used to determine sample purity with 1.85-2.15 considered free of contaminants.
2.3.2 Gel electrophoresis of nucleic acids

Small DNA fragments (<2 kb) were resolved by horizontal electrophoresis in a 1% agarose (Invitrogen cat no. 16500-500) 1 × TBE gel with 0.5 μ g/ml ethidium bromide (VWR cat no. 443922U). 5 × DNA loading buffer (50% glycerol, 5 mM EDTA, 0.3% Orange G) was added to each sample to 1 × before gel loading. Unless stated otherwise, 500 ng of 1 kb plus DNA ladder (Invitrogen cat no. 10787018) was used as a reference and gels were visualised on a UV transilluminator.

DNA fragments >2 kb were resolved by horizontal electrophoresis in a 0.7% agarose 1 × TPE gel with 0.5 μ g/ml ethidium bromide. Samples were prepared in 1 × DNA loading buffer before loading. 500 ng of 1 kb ladder (NEB cat no. #N0552) and 0.4 μ g of 2.5 kb molecular ruler (Biorad cat no. #1708205) were used as DNA size markers. Gels were run at low voltage <35 V for 14-18 hours with recirculating buffer (Loening, 1969).
2.3.3 Plasmid purification

Single *E. coli* colonies from plasmid antibiotic containing agar plates were swabbed and incubated in 5 ml L-broth >6 hours 37 °C in a shaking incubator (250 rpm). Either a Qiaprep Miniprep Kit (Qiagen cat no. 27104) or E.Z.N.A Plasmid Mini Kit (Omega cat no. D6942-01) were used to purify >20 μg plasmid DNA from 5 ml *E. coli* cultures as per manufacturers’ instructions and eluted into 30 μl TE buffer, and concentration measured on a Nanodrop 1000 UV-VIS spectrometer (Thermo Scientific). For 20-200 μg of plasmid DNA, a 5 ml *E. coli* culture was added to 200 ml L-broth overnight at 37 °C in a shaking incubator (250 rpm). Plasmid from 50 ml of culture was isolated using E.Z.N.A Plasmid Maxi Kit (Omega cat no. D6922) following the manufacturer’s instructions and eluted into 50 μl TE. For larger plasmid amounts the E.Z.N.A Plasmid Maxi Kit was used in series, as per manufacturer’s instructions.

2.3.4 Restriction digestion and ligation of DNA

Plasmid DNA was digested with the required restriction endonucleases (New England Bioloabs) following manufacturer’s guidelines. 10-20 units of restriction enzyme was typically used per 1 μg of plasmid DNA at 37 °C for 2-12 hours. T4 DNA ligase (Cat No M0202S) was used to join DNA fragments. A 3:1 vector to insert ratio was used for ligation reactions, with 10 U T4 ligase per 200 ng of plasmid. Ligation reactions were incubated at room temperature of 4 hours.
2.3.5 Restriction digested DNA isolation

DNA fragments from restriction digestions were resolved by agarose gel electrophoresis with sybr safe (Invitrogen cat no. S33102). 1 μl of a 1:10 dilution of sybr safe stock was added to each sample before loading into each lane. Gels were viewed under blue light and a single edged razor blade was used to excise the desired DNA fragment. Gel fragments where transferred to 1.5 ml tubes and the DNA extracted with QIAquick Gel Extraction Kits (Qiagen cat no. #28704) as per manufacturer’s instructions.
2.3.6 Cross linked chromatin immunoprecipitation

Approximately $5 \times 10^7$ cells per ChIP were crosslinked with 1% formaldehyde (Sigma cat no. #F87750) in FCS free media for 10 minutes at room temperature. To stop crosslinking, formaldehyde was removed and cells were washed three times with ice-cold PBS containing 125 mM glycine for 5 minutes. Cells were removed from tissue culture flasks or 10 cm petri dishes using a cell scraper and centrifuged at 500 g for 4 minutes at 4°C. The supernatant was removed and the pellet resuspended in RIPA buffer and sonicated in 1 ml sequential batches. Fragmentation by sonication was performed on a Soniprep 150 for 15 cycles 20 s on and 40 s off at amplitude of 8 μm on ice.

Samples were centrifuged at 20,000 g for 20 minutes at 4 °C to pellet large cellular debris. Supernatant was collected and a 50 μl aliquot used to check fragmentation efficiency. The 50 μl aliquot was diluted with 50 μl cell lysis buffer (85 mM KCl, 0.5% NP-40, 5 mM HEPES pH 8.0) and 5 M NaCl at 65 °C for 4 hours to reverse the formaldehyde crosslinks and incubated with 1 μl RNase A/T1 (Ambion cat no. EN0551) at 37 °C for 30 minutes. Proteinase K was added and incubated at 55 °C for 1 hour and samples were analysed by gel electrophoresis. Only samples with DNA fragments between 100-1000 bp were used for immunoprecipitation.

The chromatin concentration of sonicated samples was measured at 260 nm on a UV-VIS Nanodrop 1000 (Thermo Scientific) and normalised across all samples to >200 ng/μl. The samples are then diluted 10 fold in ChIP dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl pH 7.5, 167 mM NaCl, and protein inhibitor cocktail) and 10% stored as input.
50 μl magnetic protein A dynabeads (Invitrogen cat no. #100-02D) per immunoprecipitation were preblocked in 5 mg/ml BSA in PBS, before antibody incubation in 500 μl ChIP dilution buffer with proteinase inhibitors for 4 hours rotating (15 rpm) at 4 °C. The Dynabeads were then washed with BSA 5 mg/ml in PBS, before incubation with samples overnight at 4 °C.

Magnets were used isolate the Dynabeads from the solution and washed with TSE-I and TSE-II buffers (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, 150/500 mM NaCl) for 5 minutes at 4 °C. Samples were further washed with LiCl buffer (0.25 M LiCl, 1% NP-40, 1% deoxycholate, 1 mM EDTA, 10 mM Tris-HCl) and TE buffer for 3 minutes at 4 °C. Chromatin was eluted from Dynabeads in 250 μl elution buffer (1% SDS, 0.1 M NaHCO₃) for 1 hour at 65 °C with occasional vortexing, before 5 M NaCl was added to each sample (20 μl) and input sample (4 μl) for a further 5 hours at 65 °C. The supernatant was then incubated with RNase A/T1 for 30 minutes at 37 °C. The samples were then treated with proteinase K (2 μl proteinase K, 10 μl 0.5 M EDTA, 20 μl 1 M Tris-HCl) at 65 °C overnight. The samples were then purified using Qiagen PCR MiniElute Kits (Qiagen cat no. 28004) as per manufacturer’s instructions. DNA concentrations were then quantified using a GE healthcare GeneQuant Pro RNA/DNA Spectrophotometer (Cat no. 80-2114-98). Samples were analyzed by qPCR.
2.3.7 ChIP qPCR

To quantify the DNA abundance in each ChIP sample, real-time PCR with LightCycler™ SYBR green I Master Mix (Roche cat no. 04707516001) was used. 10 μl reactions were analysed for each sample with 0.4 μM of each primer in technical triplicates. A lightcycler 480 system was used to perform the PCR with 45 cycle amplification reactions as per SYBR green I Master Mix manufacture instructions (95 °C for 3 minutes, 45 × 95 °C for 15 s, 58 °C for 15 s, and 72 °C for 30 s). Melting curves were observed to ensure single annealing steps for each primer, with PCR products checked by 2% agarose gel electrophoresis. Ct values were used to determine amplification compared to input for each sample and antibody, using the ΔΔCt method.

2.3.8 Sanger sequencing DNA

Sanger sequencing of plasmids to check sequences were performed by the MRC HGU Technical Services using standard methods. BigDye (Thermo Fisher cat no. 4337455) was used in terminator sequencing reactions on an ABI 3730XL. Sequencing data was examined using Snapgene.
2.3.9 Nuclei preparation

To isolate nuclei for downstream experiments, Cereghini and Yaniv’s, (1984) method was modified. Cells were washed with PBS before Trypsin/EDTA (Invitrogen cat no. 59427) was used to detach them for harvest. Trypsin was inhibited by adding 10 ml of 10% FCS/PBS to each sample. Samples were centrifuged at 1200 rpm for 3 minutes, and the pellet resuspended by pipette in 1 ml of NBA buffer (85 mM KCl, 5.5% sucrose, 10 mM Tris-HCl pH 7.6, 0.5 mM spermidine, 0.2 mM EDTA, and 250 μl PMSF) on ice. A further 4 ml of NBA buffer was then added. 5 ml of NBB buffer (NBA + Δ% NP-40) was added to each sample on ice for 4 minutes. The nuclei were quickly pelleted by centrifugation 2000 rpm for 4 minutes, to prevent lysis of the nuclei. The nuclei pellet was resuspended in 10 ml of NBR2 buffer (85 mM KCl, 5.5% sucrose, 10 mM Tris-HCl pH 7.6, 1 mM CaCl₂, 1 mM MgCl₂, and 250 μl PMSF) and quickly pelleted again by 2000 rpm for 4 minutes. The nuclei pellet was then resuspended in 500 μl NBR2 buffer.

5 μl aliquot of each sample was then added to 95 μl NBR2 and 1 μl DNaseI for 4 minutes at room temperature. 400 μl of 5 M Urea / 2 M NaCl was added to each sample and quantified on a GeneQuant Pro spectrophotometer at 260 nm. Nuclei samples were normalised to 20 A260 units. 20 A260 units is equivalent to a DNA concentration of 1 mg/ml.
2.3.10 Southern Blotting

DNA was transferred from 0.7% agarose TPE gels to Hybond N+ membranes (GE Life Sciences cat no. 25005990) as first described by Southern, (1975). Agarose gels containing DNA were first exposed to UV light from a transilluminator for 3 minutes to cause double strand breaks and trimmed to size using a razor blade. Gels were incubated in 250 ml 0.25 M HCl for 20 minutes shaking at room temperature to depurinate the DNA. The gel was washed twice with 250 ml H2O to remove residual HCl and incubated twice with 250 ml denaturing buffer (1.5M NaCl, 0.5M NaOH) for 15 minutes at room temperature shaking. Once the denaturation buffer was removed and the gel rinsed with H2O, gels were twice soaked in 250 ml neutralisation buffer (1.5 M NaCl, 0.5 M Tris-HCl). Upward capillary transfer was used to transfer DNA from the gel to the Hybond N+ membrane using 20 × SSC solution overnight at room temperature. The Hybond N+ membrane was rinsed in 2 × SSC solution and exposed to UV on a transilluminator to immobilise the DNA.

[ α-32P] dCTP radiolabelled probes were then hybridised to the Hybond N+ membrane. Each membrane was incubated in 15 ml of hybridisation buffer (6 × SSC, 10 mM EDTA, 5 × Denhardt’s solution (Thermo Fisher cat no. 750018), 0.5% SDS, 100 µg/ml salmon sperm DNA) for 30 minutes. Radiolabelled probe was then added to the hybridisation solution and incubated at 65°C overnight. Membranes were washed twice with 2 × SSC 0.1% SDS for 15 minutes at 65°C followed by two washes with 0.1 × SSC 0.1% SDS at 65°C for 15 minutes. Hybridised radiolabelled probes were then detected in autoradiography cassettes with phosphorimager screens overnight and analysed.
2.4 RNA methods

2.4.1 RNA purification

Total RNA was isolated from mammalian cell cultures using Qiagen’s Rneasy Mini Kit (Qiagen cat no. 74104). Cells were washed with PBS, harvested by trypsinisation and counted using a Beckman Coulter Counter. More than $1 \times 10^7$ cells were pelleted per sample. Cell pellets were resuspended in 350-600 $\mu$l of RLT buffer with 1% $\beta$-mercaptoethanol depending on cell number. An equal volume of 70% ethanol was then added to the samples and mixed by pipetting.

Samples were transferred to Rneasy mini spin column and centrifuged at 12,000 g for 30 s. 10 $\mu$l of DnaseI was mixed with 70 $\mu$l of RDD buffer (Qiagen RNase-Free Dnase Set; Cat no. 79254) and added to each column for 15 minutes at room temperature, for on column DNase digestion. RNA was eluted from the column in 50 $\mu$l of RNase-free water, quantified on a Nanodrop-1000 UV-VIS spectrometer and stored at -80°C.
2.4.2 cDNA library synthesis

Complementary DNA to first strand RNA was synthesised using SuperScript II Reverse Transcriptase (Invitrogen cat no. 18064-022). 1 μg of purified RNA was added to 250 ng of random hexamers, and 10 mM dNTP mix in RNase free water. Samples were the incubated at 65 °C for 5 minutes to melt secondary RNA conformations and quickly cooled on ice. SuperScript II buffer was added to each sample (1 × final conc First Strand Buffer, 0.1M DTT). Samples were gently mixed and incubated at 25 °C for 2 minutes. 200 units of SuperScript II reverse transcriptase was added into each sample and incubated at 25 °C for 10 minutes, followed by 42 °C for 50 minutes. Reactions were stopped by denaturation at 70 °C for 15 minutes.

2.4.3 Quantitative real time PCR

To evaluate the relative abundance of complementary DNA sequences indicative of relative gene expression, real time PCR was used. Lightcycler 480 SYBR Green I Master Mix (Roche cat no. 04707516001) was as described earlier for analysing ChIP DNA sequences (Section 2.3.6).
2.4.4 EU-RNA dot blotting

Mouse ESCs were incubated with 1 mM ethynyl uridine (EU) overnight at 37 °C 5% CO₂. Cells were rinsed 3 × with CSK buffer (100 mM NaCl, 0.1% Triton X-100, 300 mM sucrose, 1 mM MgCl₂, 1 mM EGTA, 10 mM PIPES and 100 μM PMSF) at room temperature for 5 minutes. Cells were then trypsinised and centrifuged at 1200 rpm for 5 minutes. mESC nuclei were isolated using NBA and NBbB buffers (Section 2.3.7). RNA was purified using Qiagens Rnasey kit (Section 2.4.1).

18 μl of RNA was added to an in vitro click reaction mix (3 μl TEA pH 7, 30 μl DMSO, 6 μl ascorbic acid, 1.2 μl biotin-azide (Thermo Fisher cat no. B10184), 3 μl 10 mM Cu-TBTA, and vortexed to ensure complete mixing. Samples were left overnight at room temperature for the click reaction to complete. RNA was precipitated using 150 μl of ethanol and 0.3 M sodium acetate pH 5.5 and 20 μg of glycogen at -20 °C for 2 hours. Centrifugation at 12,000 rpm for 30 minutes was used to pellet the RNA and samples were washed in 500 μl 70% ethanol. Samples were pelleted again and left to air dry to remove residual ethanol at room temperature. Samples were resuspended in 20 μl TE and the absorbance measured using a nanodrop 1000 UV-VIS spectrometer.

Nitrocellulose membranes were hydrated in water for 5 minutes followed by 20 × SSC for 20 minutes at room temperature. Excess 20 × SSC buffer was absorbed using filter paper and 2 × 1 μl of RNA TE was then spotted and dried at room temperature. The RNA was crosslinked to the membrane with 200 mJ of UV radiation and baked at 70 °C for 30 minutes.
The crosslinked membranes were then blocked with 50 ml of 3% BSA (w/v) in buffer 1 (0.5 M Tris, 0.15 M NaCl) at 60 °C for 30 minutes. For biotin detection membranes were incubated with Streptavidin-HRP (Thermo Fisher cat no. 89880D) at 1:1000 dilution for 2 hours rotating at room temperature. Membranes were washed twice with buffer 1 for 15 minutes with shaking to remove any unbound streptavidin-HRP molecules. Membranes were then developed with enhanced chemiluminescence detection kit (Pierce cat no. 32106) for 1 minute at room temperature before exposure to Hyperfilm ECL (GE Healthcare cat no. 28906837).
2.5 Protein analysis

2.5.1 Protein lysate preparation

Proteins were extracted from mammalian cell cultures by detergent mediated cell lysis. Cells were typically grown in T75 flasks or 6 cm dishes to 75% (max) density. Cells were washed with PBS and recovered by trypsinisation (Invitrogen cat no. 59427C). Non-adherent cells were collected and lysed with NuPage LDS sample buffer (Invitrogen cat no. NP0007) with 12.5 mM DTT as per manufacturer’s instructions. Samples were incubated at 70 °C for 10 minutes before being stored at -20 °C.

2.5.2 SDS-PAGE

Nupage Novex precast gels were used to resolve protein lysates. Nuclear lysates were centrifuged at 12,000 g for 1-2 minutes to pellet any large cellular fragments. 10-20 μg was loaded per well on 4-12% gradient (Invitrogen cat no. NP0322BOX) or 10% (Invitrogen cat no. NP0315BOX) Bis-Tris gels. 4 μl of SeeBlue Plus2 prestained protein standard (Invitrogen cat no. LC5925) was run to determine molecular weights. For low molecular weight proteins such as histones, 1 × Nupage MES running buffer (Invitrogen cat no. NP0002) was used, while 1 × Nupage MOPS running buffer (Invitrogen cat no. NP0001) was used for high molecular weight proteins (>80 kDa). Proteins were resolved at 150 V for 50 minutes with MES buffer, and 80 minutes with MOPS running buffer.
2.5.3 Western blotting

Nuclear protein lysates resolved by SDS-PAGE electrophoresis were transferred to Immobilon-P polyvinylindine flouride (PVDF) membranes (Millipore cat no. IPVA00010). ‘Wet’ transfers were performed using the X Cell 11 Blot Module (Invitrogen cat no. EI9051). Blotting pads and filter paper were submerged in 1 × Nupage transfer solution (Invitrogen cat no. NP0006) supplemented with 20% methanol, 1% antioxidant (Invitrogen cat no. NP0005) and any air bubbles removed. PVDF membrane was cut to size and hydrated in 100% methanol before being soaked in transfer solution. Protein transfer was performed at 30 V for 60 minutes.

The protein bound PVDF membrane was incubated in 5% milk powder TBS-T buffer (50 mM Tris HCl pH 7.5, 150 mM NaCl, 0.05% Tween-20) for 30 minutes shaking at room temperature. PVDF membranes were washed with TBS-T for 10 minutes at room temperature. Membranes were then incubated with primary antibodies in 5% milk PBS-T overnight at 4 °C.

Primary antibody was removed and the membrane washed 3 × with TBS-T shaking at room temperature for 5-10 minutes. Antibody species specific conjugated horseradish peroxidase (HRP) secondary antibodies were diluted in 5% milk TBS-T and added to the membranes while shaking at room temperature for 1 hour. Membranes were briefly washed in TBS-T and incubated with an enhanced chemiluminescence detection kit (Pierce cat no. 32106) for 1 minute at room temperature. Membranes were place between acetate sheets and exposed to Hyperfilm ECL (GE lifesciences cat no. 28906837) and developed on a Konika SRX-101A processor. Films were scanned and densitometry performed on the required bands using ImageJ or AIDA image analysis software with background correction.
2.6 Cell Culture

2.6.1 Cell lines

Cell lines used were murine erythroleukaemia cells (Chapter 3), HCT116 cells and matched trametinib resistant TRAMR cells (Chapter 4), murine 3T3 fibroblasts, and mouse embryonic stem cells wild type (WT41) and Suv39h double null clones (DN57, DN72) (Chapter 5).

MEL cells with various GFP constructs inserted at the RL5 locus were obtained from the Larionov lab. p212 MEL cells lacked any heterochromatic sequences within the transgene, while RL5 MEL cells lacked the GFP construct entirely. α-MEL cells contained a human alpha satellite domain upstream of the GFP enhancers and promoter (Figure 3.1, A). HCT116 cells and matched trametinib resistant TRAMR (derived from HCT116) cells were obtained from John Dawson, who initially characterised the cellular phenotype. Mouse embryonic stem cells were obtained from the Jenuwein lab.
2.6.2 Adherent cell growth and passaging

HCT116, TRAMR and 3T3 cells were typically cultured in T75 flasks with 15 ml of Dulbecco’s Modified Eagle Medium F12 (Gibco cat no. 12500-062) supplemented with 10% fetal calf serum, 1% penicillin/streptomycin and 1% L-glutamine. Cells were grown in 5% CO₂ at 37 °C in a humidified cell culture incubator. When confluent (> 70% coverage) cells were passaged by washing with PBS and treated with trypsin/EDTA (Gibco cat no. 5400054) and split 1:7 to 1:10 every 2-3 days.

2.6.3 Suspension cell growth and passage

MEL cells were also cultured in T75 flasks with 20-30 ml high glucose Dulbecco’s modified eagle medium with L-glutamine (Gibco cat no. 11965084) supplemented with 10% fetal calf serum (FCS) and 1% penicillin/streptomycin. Cells were grown at 5% CO₂, 37 °C and split 1:10 to 1:20 for every other day.
2.6.4 mESC cell growth and passage

Mouse embryonic stem cells were only grown with low passage numbers (<50). WT41, DN57, and DN72 cell lines were cultured in Glasgow Minimal Essential Medium (Gibco cat no. 11710035) supplemented with 10% fetal calf serum, 0.1 mM β-mercaptoethanol, 1% penicillin/streptomycin, 1% L-glutamine, 1 mM sodium pyruvate, non-essential amino acids (Sigma cat no. M7145), and 1000 U/ml of human recombinant LIF (prepared in MRC HGU). mESC flasks (Corning cat no. 430639) were pre-treated with 0.1% gelatin/PBS for 5 minutes. The excess gelatin was removed and 5 ml of mESC media was added to prevent the gelatin layer drying out. mESCs suspended in 20 ml of media were added to each flask.

2.6.5 Freezing cells

For cell storage, >80% confluent cell flasks were washed with PBS, incubated with trypsin/EDTA (Gibco cat no. 5400054) for 5 minutes, collected by centrifugation at 1200 rpm for 5 minutes. Cells were resuspended in 1-2ml freezing media (50% fetal calf serum, 10% DMSO, 40% ES cell growth media). 500 μl aliquots were frozen at -80°C and transferred to liquid nitrogen after 24 hours for long term storage.
2.6.6 Thawing cells

Cell lines retrieved from liquid nitrogen or -80°C storage were kept on dry ice before being rapidly thawed at 37 °C in a water bath. Once thawed the contents was added to 10 ml of normal growth media pre-warmed to 37 °C and centrifuged at 1200 rpm for 5 min. Cell pellets were resuspended in 15 ml of growth medium in a T75 flask stored at 5% CO₂ and 37 °C. After 12-24 hours flasks were rinsed with PBS and 15 ml fresh growth media added. Media was replaced every 1-2 days and cells passaged every 72 hours or whenever mESC colonies were enlarged.

2.6.7 Stable mammalian cell transfection

Mouse embryonic stem cells were seeded at 50,000 cells/ml on 0.1% gelatin coated 6-well plates overnight at 37 °C in antibiotic free growth media. 1 μg of the vector construct was incubated with 200 μl of Opti-MEM (Gibco cat no. 31985070) for 10 minutes, whilst 5 μl lipofectamine 2000 (Invitrogen cat no. 11668019) was added to a separate 200 μl Opti-MEM aliquot per transfection. The pre-mixed lipofectamine 2000 and DNA constructs were mixed by inversion and incubated at room temperature for 30 minutes. This mix was then added to 2 ml of fresh antibiotic free media and used to refresh the media on a mESC culture. mESC’s were then grown with appropriate antibiotic (Zeocin) prior to experiments.
2.6.8 Generating drug resistant cell lines

Cells were initially treated with log dose curves of JQ1 for 5 days and viability determined by PrestoBlue reagent (Invitrogen cat no. A13261). Cells were seeded at 10,000 cells/ml in 96 well plates with 200 μl of media in each well. Cells were left over night to adhere to the well bottom, and the media was then aspirated and replaced with JQ1 containing media for 4 days. The media was removed and fresh media with 10% PrestoBlue (v/v) was added to each well for 90 minutes. 540/590 nm fluorescence was assayed on a PerkinElmer Victor3 multilabel reader.

The concentration of JQ1 that inhibited growth by 50% (195 nM) was then added to the growth media for two weeks. Cell viability in response to a log dose concentration of JQ1 was then reanalysed with PrestoBlue reagent, and the JQ1 dose adjusted for the next two-week period. After ~3 months cells could tolerate 1 μM JQ1 treatment and were considered resistant. JQ1 was then removed from the growth media and viability re-analysed after two weeks. Cells presented no loss of resistance to JQ1 demonstrating a permanent state of resistance. Cells were periodically retested to check JQ1 resistance was not lost.
2.6.9 Immunofluorescence

To analyse the cellular distribution of proteins and their modifications cells were immunostained with appropriate antibodies. Cells were seeded on Superfrost plus slides (Thermo Fisher cat no. J1800AMNZ), pre-coated in 0.1% gelatin for mESC’s, for 6-8 hours at 37 °C 5% CO₂ in chambers with 5 ml of growth media.

To observe chromatin bound protein distribution slides were treated with CSK buffer (100 mM NaCl, 0.1% Triton X-100, 300 mM sucrose, 1 mM MgCl₂, 1 mM EGTA, 10 mM PIPES and 100 μM PMSF) for 10 minutes on ice.

Slides were carefully washed with PBS to prevent loss of adherent cells and fixed with 4% paraformaldehyde (Sigma cat no. 158127) for 10 minutes at room temperature. Slides were carefully removed and submerged in PBS twice to remove any residual paraformaldehyde and permeabilised in 0.5% Triton X-100 (Sigma cat no. T8787) for 10 minutes at room temperature.

Slides were then blocked in 5% donkey serum in PBS for 30 minutes at room temperature. Primary antibodies were then added to the slides diluted in 5% donkey serum and incubated for 1 hour at room temperature in a dark humidified chamber. Slides were washed 3 × in PBS/ 0.02% Tween-20 5 minutes each at room temperature. Fluorophore conjugated secondary antibodies (Jackson Immuno Research) were incubated on the slides for 1 hour at room temperature in a dark humidified chamber, diluted 1:500 with 5% donkey serum/PBS. Slides were kept in the dark and washed carefully 3 × with PBS 0.02% Tween-20 for 5 minutes at room temperature. For nuclei staining the final wash was supplemented with 50 μ g/ml of DAPI at room
temperature. Slides were mounted in Vector Shield (Vector Laboratories cat no. H-1000). Standard and deconvolution epifluorescent microscopes using 63x or 100x plan-APOCHROMAT (1.4 NA) objectives were used to image slides. Deconvolution analysis was performed using iVision software.
2.6.10 Chromatin bound protein distribution analysis

To determine chromatin bound protein distribution, samples were washed with CSK buffer prior to fixation and imaged on a deconvolution epifluorescent microscopes as stated above (Section 2.6.8). Image stacks were initially deconvolved on iVision software per standard protocols. Image stacks were then transferred to ImageJ. A macro in Javascript was written to split the channels, identify the single plane for analysis, restack the channels for the plane of interest, and save the stack.

A second macro was written to use edge segmentation to make a mask of the DAPI channel. The DAPI channel was first applied with a rolling ball background subtraction, unsharpen mask to rescale the contrast to the original image, and processed with a gaussian blur to smooth the image further. The image was then made binary with a black background. The classic watershed function was applied to edge segment the image and particles under 500 pixels excluded. This created the region of interests (ROI) around each nucleus in each image. This mask was then applied to the FITC channel, where the area mean integrated intensity for each pixel was calculated within the ROI, determining the radial pixel intensity and exported to excel.

Each radial pixel number was divided by the max radial pixel number per ROI and multiplied by 100 to turn pixel number in percentage distribution. This enabled the data of multiple cells to be binned together into radial decile pixel intensities, which were then plotted by box plot.
2.6.11 Cell cycle analysis with propidium iodide

Propidium Iodide (PI) is a DNA intercalator that has 20 times the fluorescence when bound to DNA (Wilson et al., 1986). As DNA content changes as a cell progresses throughout the cell cycle, PI staining was used to investigate cell cycle dynamics. Cell cultures were first washed with PBS and harvested by trypsinisation. Cells were centrifuged at 1200 rpm for 5 minutes, resuspended in PBS and counted using a Z2 Coulter counter. Cell counts were normalised to $1.5 \times 10^6$ cells/ml and 2.5 × volume of 100% ethanol was pipetted dropwise while samples were vortexed. Cells were incubated on ice for >2 hours to allow fixation, centrifuged at 2000 rpm for 5 minutes at room temperature and resuspended in PBS. Samples were then recentrifuged and cells resuspended in propidium iodide staining solution (1 $\mu$g/ml PI, 4 $\mu$g/ml RNase A, PBS) at a count of $2.5 \times 10^6$ cells/ml for 2 hours at room temperature.

Flow cytometry was used to measure PI staining on a BD LSR Fortessa cell analyser. Filters limited fluorescence detection to 695-740 nm. Data analysis of cell population was performed using Flow-Jo single-cell flow cytometry software.
2.7 Radioactive nucleotide labelling

2.7.1 DNA probe labelling

25 ng of oligomers or DNA molecular ladders in 11 $\mu l$ DNase free water were boiled for 5 minutes and placed on ice to cool. 4 $\mu l$ of High Prime Kit (Roche cat no. 11585584001) supplemented with 125 mM dATP, dGTP, and dTTP was added to each sample as per manufacturer instructions. 5 $\mu l$ of [$\alpha$-$^{32}$P] dCTP 3000 Ci/mmol was also added and incubated at 37 $^\circ$C for 30 minutes. 80 $\mu l$ of DNase free water was added to each sample and transferred to a G-25 sephadex spin column (Roche cat no. 27532501).

2.7.2 Unincorporated label removal

Unincorporated free nucleotides dATP, dGTP, dTTP and [$\alpha$-$^{32}$P] dCTP were removed from DNA probes by passing through sephadex G-25. Columns were prepared by vortexing and then the cap loosened ¼ turn and centrifuged $>$700 g for 1 minute. 100 $\mu l$ of radiolabelled samples were then carefully transferred to the column without disturbing the G-25 grade F resin. Samples were then centrifuged $>$700 g for 2 minutes at room temperature. The [$\alpha$-$^{32}$P] dCTP labelled DNA was recovered in the eluate and stored for hybridisation.
2.7.3 RNA metabolic labelling

Cells were grown in T75 flasks to 60% confluency and treated with JQ1 1 μM for 24 hours. 5 μl/ml of 1 mCi [5-3H] Uridine (Perkin Elmer cat no. NET174250UC) was added to the culture media for 30 minutes at 37 °C 5% CO2. Media was aspirated from the cells and washed with PBS. Cells were trypsinised, collected and washed again with PBS. RNA was extracted from the cells using the RNeasy Mini Kit as previously described (Section 2.4.1). Samples were quantified on a nanodrop 1000 UV-VIS spectrometer 260 nm absorbance and 5 μl of each sample was then added to 4 ml of scintillant. Radiation from the tritiated RNA causes the scintillant to fluoresce allowing the quantity to be analysed using a Beckman LS 6500 scintillation counter. Samples were counted for 30 minutes at room temp in technical triplicates.
2.8 Chromatin analysis

2.8.1 Chromatin fibre extraction

In order to release chromatin fibres from isolated nuclei, chromatin was partially digested with micrococcal nuclease (Mnase). Nuclei at 20 A260 in NBR2 buffer were incubated with RNase A/T1 (Ambion cat no. EN0551) for 2 minutes at room temperature. Each sample was then digested with 400 units/ml of MNase for 8 minutes at room temperature. 125 mM glycine final concentration was used to stop the MNase digestion, and samples were quickly pelleted at 12,000 g for 1 minute and resuspended in 500 μl TEEP-20 or TEEP-80 buffer at 4°C overnight. Samples were centrifuged at 12,000 g for 5 minutes at room temperature to remove nuclear fragments from the supernatant.

Samples fixed with 1% formaldehyde for 10 minutes before or after MNase digestion, were released into TEEP-20 (+/- 0.5% SDS) and transferred to Visking tubing. The samples were then dialysed in 5 litres TEEP-80 or TEEP-1 at 4 °C overnight to remove excess formaldehyde molecules.
2.8.2 Chromatin purification – Sucrose step Gradients

Chromatin fibres and chromatin bound proteins can be isolated from nuclear components by centrifugation onto a TEEP-80 50% sucrose boundary as described in Gilbert, et al, (2007). A 1.5 ml layer of 50% sucrose TEEP-80 was added to a SW55 Beckman tube. 10% sucrose TEEP-80 was then slowly layered on without mixing, resulting in a boundary between the 10-50% sucrose. Chromatin samples released from nuclei were loaded on the 10% TEEP-80 and centrifuged 48,000 rpm for 90 minutes. 60% sucrose TEEP-80 was pumped in the bottom of the centrifuge tubes, using upward displacement to fractionate the gradient into 500 μl aliquots. DNA from chromatin containing fractions was then purified by phenol chloroform extraction.

2.8.3 Isokinetic sucrose gradient fractionation

Sucrose density centrifugation is a technique used to fractionate macromolecules that has been adapted to distinguish between chromatin states (Brakke, 1953; Gilbert, Thomson, Boyle, Allan, Ramsahoye and Wendy A Bickmore, 2007). Micrococcal nuclease released chromatin fibres were resolved in 6-40% isokinetic sucrose TEEP gradient centrifugation in Beckmann SW41 tubes. 6% sucrose TEEP Δ NaCl was mixed with 40% sucrose TEEP Δ NaCl solution at room temperature in a constant volume mixing chamber and loaded bottom up as first described by Noll, (1967). Gradients were then left overnight at 4 °C to settle. 0.5 cm was left free at the top of each SW41 tube sucrose gradient, and 500 μl of soluble chromatin was loaded. Samples were centrifuged in a Beckman ultracentrifuge using a
SW41 Ti swinging bucket rotor at 41,000 rpm, 4 °C for 4.5 hours. Gradients were fractionated using upward displacement with 50% sucrose TEEP-80 and samples were passed through an inline UV spectrometer and collected in 500 μl aliquots. The UV absorbance was used to detect which fractions contained chromatin fibres, which were then purified by phenol chloroform. DNA samples were fractionated on 0.7% agarose TPE gels.

2.8.4 Micrococcal Nuclease accessibility digestion

To assess micrococcal nuclease accessibility, isolated nuclei (Section 2.3.7) were diluted to 4 A260 (200 μg/ml) in NBR2 buffer. Samples were incubated with 1 μl of RNase A/T1 for 2 minutes at room temperature. 800 units/ml of micrococcal nuclease was then added to the samples at room temperature. At 1, 2, 4, 8, and 16 minutes aliquots were added to EDTA to stop the MNase digestion. Each time point sample was then added to genomic lysis buffer 1 × final concentration and incubated with 1 μl proteinase K at 55 °C overnight. Samples were phenol chloroform purified and ethanol precipitated before being resolved on 1% agarose TBE gel with a 1 kb plus ladder.
2.9 Reverse phase protein arrays

α-MEL cells were treated with 0.1% DMSO, 1 μM IBET151 for 24 hours in normal cell culture conditions. Cells were washed in PBS, trypsinised and resuspended in MD anderson buffer (1% Triton X-100, 50 mM HEPES, 150 mM NaCl, 1.5 mM MgCl₂, 1 mM EGTA, 199 mM NaF, 10 mM Na pyrophosphate, 1 mM Na₃VO₄, 10% glycerol, phosphatase inhibitor cocktail - Roche cat no. 04 906 845 001) for 30 minutes at room temperature with intermittent vortexing.

Protein concentrations were analysed by Coomassie Plus staining (Pierce cat no. 23236). A BSA serial dilution was used as a standard (0, 0.05, 0.1, 0.15, 0.2, 0.3, 0.4, and 0.6 mg/ml). 2.5 μl of each sample were diluted 1:20 to 50 μl and transferred to opaque 96 well plates in addition to 10 μl of standards. 240 μl of Coomassie Plus reagent was added to each well for 30 s while shaking and further incubated at room temperature for 10 minutes. 595 nm filter microplate reader was then used to measure absorbance. Lysates were normalised to 1 mg/ml and 150 μl aliquots transferred to fresh tubes.

50 μl of 4x SDS buffer was then added to each sample (40% glycerol v/v, 8% SDS v/v, 0.25 M Tris-HCl pH 6.8, 5 mM β-mercaptoethanol). Samples were incubated at 80 °C for 5 minutes and rapidly cooled by 15 s max speed centrifugation. Samples were then transferred to a 96 well plate in duplicate. Samples were half log serial diluted with MD Anderson lysis buffer (1% Triton X-100, 50 mM HEPES, 150 mM NaCl, 1.5 mM MgCl₂, 1 mM EGTA, 199 mM NaF, 10mM Na pyrophosphate, 1 mM Na₃VO₄, 10% glycerol, phosphatase inhibitor cocktail -Roche cat no. 04 906 845 001) supplemented
with 1 × SDS buffer (2% SDS, 62.5 mM Tris-HCl pH 6.8, 1.25 mM β-mercaptoethanol). Samples were spotted onto arrays at 70% humidity using an Aushon 2470 microarray printing platform and samples were left at room temperature 70% humidity for 2 hours to allow the samples to dry. Once the arrays were removed from the printing platform, they were incubated with SuperBlock TBS (Pierce cat no. 37535) for 4 hours at room temperature on a rocker.

Each array was placed in ArrayIt hybridisation cassettes and rinsed 3 × with 130 μl TBS-T, followed by incubation with TBS-T at room temperature for 10 minutes. Primary antibodies diluted in SuperBlock were added to the array cassettes and incubated overnight at 4 °C with rocking. The cassettes were rinsed 3 × with TBS-T and incubated 3 × with TBS-T for 10 minutes. Secondary antibodies and IgG control were incubated in the array cassettes for 1 hour at room temperature in the dark. The array cassettes were then further rinsed 3 × with TBS-T and submerged in 50 ml of dH₂O. The arrays were then spun at 200 g for 5 minutes to remove any residual water and left overnight in the dark at room temperature. Arrays were imaged on an Innopsys Innoscan 710 using 785 nm excitation.
Sample spotting concentrations were quality controlled and normalised for analysis using fast green staining. Arrays were first washed in 50 ml dH₂O for 5 minutes at room temperature before incubation with 0.25 M sodium hydroxide for 15 minutes and washed 3 × in dH₂O for 10 minutes at room temperature. Arrays were then incubated in destain solution (30% methanol, 7% glacial acetic acid, dH₂O) for 15 minutes at room temperature. Arrays were incubated with 1x Fast Green destain solution (250 μg Fast Green FCF (Sigma cat no. F7252), 30% methanol, 7% glacial acetic acid, dH₂O) for 3 minutes at room temperature with shaking. Arrays were submerged 3 × in 50 ml dH₂O and centrifuged at 200 g for 5 minutes to remove residual water. Arrays were scanned again with InnoScan 710 at 785 nm.
2.10 Compound screening

2.10.1 FACS screening

MEL cells were washed with PBS and concentration analysed by a Coulter counter. Cell concentrations were normalised to 333,000 cells per ml in fresh media and 300 μl added to each well of a 96 well plate resulting in 100,000 cells per well. Cells were incubated at 37 °C in 5% CO₂ for 6 hours before compounds were added.

Compounds were first diluted to 1000 × final concentration in DMSO (1 mM unless stated otherwise) in V-bottomed 96 well plates in the required final layout. Plates were sealed and kept at 4 °C in the dark for replicate use.

The 1000 × final concentration (1 mM) compounds were then diluted 100 × in fresh growth media in an intermediary 96 well plate. 33 μl of the 10 × probe compounds were added to 300 μl of MEL cell growth media in each well. Cells were incubated for 48 hours at 37 °C in 5% CO₂ before being pelleted at 1200 rpm for 5 minutes and resuspended in 100 μl ice cold PBS. Cells were kept on ice and immediately analysed on a BD FACSARia flow cytometer analyser. Samples were initially gated on side scatter height and area to ensure only single cells were analysed. Forward scatter and side scatter area was gated to remove cellular debris. GFP transgene expression was determined by 488 nm excitation and 525-550 nm emission filters. RFP expression was determined by 561 nm excitation and 610-620 nm emission filters.
2.10.2 IncuCyte growth screening

MEL cells with GFP and RFP expression constructs were quantified on a coulter counter. Cells were seeded in clear-bottomed 96 well plates at 50,000 cells/ml in 180 μl of prewarmed growth media. Each well was supplemented with 20 μl of 2 mg/ml of Corning matrigel (Cat no. 354263), previously stored at 4 °C was added to each well of 96 well plates. Plates were inserted into an IncuCyte Zoom microscope at 37 °C and 5% CO₂.

Matrigel is the base components from Engelbreth-Holm-Swarm mouse sarcoma extracellular membrane extract (Laminin, Collagen IV, Heparin sulfate proteoglycans, entactin/nidogen). As the matrigel warms to 37 °C it solidifies to form a hydrogel preventing the MEL cells from moving between focal planes allowing live cell imaging. The IncuCyte Zoom was used to image bright field, GFP and RFP every 6 hours for the length of the experiment. Four 10 × images were taken per well per time and collated.

Probe compounds were then prepared as 10 × final concentrations in fresh growth media as described for FACS screening (Section 2.10.1). After 12 hours at 37 °C, 22 μl of 10x concentration probe compounds were then added to each well. Cells were grown at 37 °C in 5% CO₂ and imaged every 6 hours up to 84 hours. Quantitative data analysis was performed using the IncuCyte Zoom analysis software. Brightfield images were used for edge segmentation, with the processing definition adjusted manually for each experiment. This mask was then applied to the GFP and RFP channels. The mean fluorescent pixel intensity for all cells within each image mask were then calculated and exported to excel. At each time point samples were compared to untreated DMSO controls.
2.10.3 Presto Blue viability assays

Single agent and drug combinations effects on viability were analysed using PrestoBlue reagent (Invitrogen cat. no. A13261). Adherent HCT116 and TRAMR cells were washed with PBS, trypsinised and concentrations adjusted to 10,000 cells/ml of growth media. 200 μl aliquots were added to each well and incubated at 37 °C overnight.

Single agent log dose concentrations were first prepared 1000 × final concentrations in V-bottomed 96 well plates. 10 mM of each compound was serial diluted to 100 μM in DMSO. Trametinib was also serial diluted from 300 μM to 100 nM. Each drug concentration was added in separate wells to every concentration of the other drug, reducing the concentration of each drug 2-fold. An equal volume of DMSO was therefore added to each single agent drug dose range to halve the concentration.

Drug matrix intermediary plates were diluted 50-fold in fresh growth media. 22 μl was added to each well of a 96 well flat bottomed plate containing cells incubated overnight. Cell were then incubated for 48 hours at 37°C in 5% CO₂. 10% (v/v) PrestoBlue reagent was then prepared in fresh growth media. Each well was then aspirated and replaced with 200 μl of 10% PrestoBlue growth media, and incubated for 90 minutes. A Victor3 multilabel plate reader was then used to detect 540/590 nm fluorescence.

Readings were normalised to DMSO control readings producing relative changes to cell growth. 3D plots of relative cell growth across the combination and antagonism/synergism analysis was performed using the combbenefit platform (Di Veroli et al., 2016). Combination matrices were
analysed in parallel using Loewe’s model, the Bliss model and the Highest Single Agent (HSA) model. Synergy, antagonisms or neutrality modelling was similar across all three models, so HSA model was used across all figures to allow direct comparison.
2.11 Copper assisted azide-alkyne cycloaddition

2.11.1 Single nucleotide HPLC-UV spectrometry

Cells were washed in PBS, trypsinised and resuspended in genomic lysis buffer (150 mM NaCl, 1% SDS, 10 mM EDTA). Samples were then incubated with 10 μl/ml proteinase K and incubated at 55 °C overnight shaking at 500 rpm. An equal volume of phenol chlorofom:IAA was then added to each sample and centrifuged at 12,000 rpm for 30 minutes at 4 °C. The aqueous phase was then transferred to a fresh tube and retreated with an equal volume of chloroform in the same fashion. The aqueous phase was added to 2.5 × volume of 100% ethanol and 0.3 M sodium acetate pH 5.5 at -20 °C for 2 hours. Samples were precipitated and collected by 12,000 rpm centrifugation for 15 minutes. The supernatant was then removed and the samples air-dried at room temperature. Samples were resuspended in 400 μl TE and 6 μl RNase A/T1 added at 37 °C overnight.

Samples were again precipitated with 2.5 × volume of 100% ethanol and 0.3 M sodium acetate pH 5.5 at -20 °C for 2 hours. Samples were pelleted and allowed to air dry, before being suspended in 400 μl TE. 6 μl RNase A/T1 was again added at 37 °C for 6 hours. Samples were re-precipitated by the same method described and resuspended in 25 μl TE. 3 μl of DNaseI and 10 × reaction buffer were added to each sample and incubated overnight at 37 °C shaking at 500 rpm. Zinc sulphate and sodium acetate were added to each sample at 1 mM and 30 mM final concentrations respectively. 3 μl of
nuclease P1 and 48 μl TE was also added and samples were incubated for 6 hours at 37 °C. Samples were then centrifuged at 12,000 rpm for 5 minutes and transferred to fresh tubes before loading onto the HPLC column. Bernie Ramsahoye ran samples on the HPLC-UV spectrometer.

### 2.11.2 Single nucleotide HPLC-MS-MS

Single nucleotide solution was prepared following the same protocol as HPLC-UV analysis (Section 2.11.1). Cells were lysed in genomic lysis buffer, and the nucleic acids isolated by phenol chloroform:IAA extraction. RNA was then digested by RNase A/T1 and centrifugation used to purify DNA molecule from the mono RNA nucleotides. The DNA was digested to mononucleotides by sequential digestions with endo- and exonucleases, DNaseI and Nuclease P1. Samples were run on a Thermo Q-Exactive Orbitrap coupled with a Dionex UPLC column, by Andrew Finch.
2.11.3 Click optimisation coumarin assay

3T3 cells were resuspended at 50,000 cells/ml and 100 \( \mu l \) added to each well of a 96 well plate. Media was supplemented with 20 \( \mu M \) of EdU for 24 hours, allowing for EdU to be incorporated in DNA during replication. 3-azido-7-hydroxycoumarin [Coumarin-azide] (Baseclick cat no. BCFA-047-5) fluorescence was used to determine the rate of azide-alkyne copper assisted cycloaddition.

Cells were incubated with 200-250 mM coumarin-azide with a dose curve of each click reaction component in turn, across the 96 well plates. EdU treated cells were supplemented with 0.25-1 mM \( \Delta \) metal (copper sulphate, ruthenium, magnesium chloride, and copper bromide), 0.01-1 mM \( \Delta \) chelating agents (THPTA, TBTA), and 0.1 mM – 1 M sodium ascorbate.

The plates were imaged using the IncuCyte Zoom, with four 10x images per well imaged every 30-60 minutes with brightfield and GFP 400 ms channels for up to 72 hours. Images were processed with edge segmentation using IncuCyte Zoom software as described before (Section 2.10.2). Total integrated intensity values for edge-segmented cells was exported to excel and plotted over time between reaction conditions.
To determine if cellular membrane disruption enhanced azide-alkyne copper assisted cycloaddition kinetics, cells were treated with optimal click conditions supplemented with Triton X-100 or lipofectamine. 3T3 cells incubated with 20 \( \mu \)M EdU for 24 hours as described above were treated with 250 mM coumarin-azide, 250 mM NaAsc, 250 \( \mu \)M THPTA, with 0.001-1\% Triton X-100 for 6 hours. 500 \( \mu \)M CuSO\(_4\) was then added to start the click reaction.

To investigate the effect of lipofection on click reaction kinetics, optimal click reaction final concentration conditions (250 mM NaAsc, 250 mM Coumarin-azide, 250 \( \mu \)M THPTA) was made at 2 \( \times \) concentration in opti-MEM for 10 minutes at room temperature. Lipofectin, Lipofectamine 2000 and Lipofectamine 3000 were separately incubated in an equal volume of opti-MEM for the same time period at room temperature. Click reaction opti-MEM was then mixed 1:1 with lipofectin, lipofectamine 2000 or 3000 and incubated on 3T3 cells for 6 hours. 500 \( \mu \)M CuSO\(_4\) was then added to each well to start the click reaction, and cells were imaged on the IncuCyte Zoom as per coumarin-azide click optimisation.
2.11.4 Naked DNA in vitro click crosslinking reaction

*E. coli* transfected with pUC57-Recon2 was grown in 5 ml of Luria-Burtani broth (L-broth) overnight in a shaking incubator (250 rpm). Cultures were then supplemented with 5 ml of additional L-broth and 20 μM of EdU for 3 hours in the shaking incubator. EdU incorporated plasmid was isolated and an aliquot digested and analysed by HPLC-UV as described above (Sections: 2.3.3, 2.11.1). pUC57-Recon2 was then digested with *Xho*I at 37 °C overnight with shaking (500rpm). Optimal live cell click conditions (0.5 mM CuSO₄, 0.25 mM THPTA, NaAsc 250 mM) and 100 mM of PEG-bis-azide ~5000 M.W. (Sigma cat no. 689580) were then added to the digested plasmid at room temperature for 1-8 hours. Samples were then resolve by 1% agarose TBE electrophoresis.

2.11.5 Live cell click crosslinking reaction

Mouse fibroblast 3T3 cells were incubated with 20 μM EdU and 100 mM PEG-bis-azide (~5000 M.W) for 24 hours in normal cell culture conditions. Optimal live cell click conditions (0.5 mM CuSO₄, 0.25 mM THPTA, 250 mM NaAsc) was then added to the growth media for 1 hour at 37 °C in 5% CO₂. Growth media was then aspirated, and cells were washed in PBS, trypsinised and resuspended in NBA for nuclei isolation and chromatin released as described previously (Sections: 2.3.7, 2.8.1). MNase released chromatin fibres were released into TEEP-20 buffer before being dialysed in Visking tubing into TEEP-80 or TEEP-1 overnight at 4 °C. Samples were then centrifuged on TEEP-80 or TEEP-1 6-40% isokinetic sucrose gradients respectively at 41,000 rpm for 4.5 hours at 4 °C as described in Section 2.8.3.
Chapter 3: Screening for heterochromatin factors

Mis-regulation of epigenetic mechanisms has been attributed to the aberrant phenotypes of numerous conditions, including cancer. The study of altered gene regulation has largely focused on gene activation, as this provides an obvious mechanism of action, and therefore a validated target for therapeutic inhibition, but gene silencing also contributes to disease phenotypes. Within a cancer setting, genes that require silencing for oncogenesis to occur are termed tumour suppressors. Expression of repetitive DNA elements from normally heterochromatic loci are also linked to genomic instability and disease phenotypes (Kazazian et al., 1988) whilst loss of pericentric heterochromatin and expression of underlying satellite repeats occurs in human and mouse tumours across cancer types (Ting et al., 2011). Therefore, identifying the fundamental mechanisms of chromatin condensation both at constitutive and facultative loci remains an important biological question, with under-appreciated potential therapeutic benefit.
3.1 A synthetic model of heterochromatic silencing

To identify factors affecting heterochromatin formation and gene silencing, an artificial P212 construct, developed by Kim et al., (2009), was used to screen a library of drugs. This system uses isogenic murine erythroleukaemia cells with different elements inserted at the Scl/Map17 (RL5) locus on chromosome 4, via the LoxP/Cre system (Feng et al., 2005). The Scl gene encodes for a transcription factor, and is expressed in pluripotent haemopoietic stem cells, and is required for haemopoietic lineage differentiation. Lineage specific DNaseI hypersensitivity sites at the locus correlate with Scl expression, and therefore regulated disruption to the chromatin fibre have been proposed to modulate local transcription (Gottgens et al., 1997; Göttgens et al., 2001). Other studies have identified a downstream CTCF site that acts as an insulator from the Cyp gene cluster expressed in the liver. While not a tissue specific enhancer, this CTCF site is required to separate Scl and Cyp regulation (Follows et al., 2012). As MEL cells have a haemopoietic lineage, the local chromatin environment is therefore considered to be normally permissive of transcription.
Figure 3.1. Organisation and dynamics of an artificial heterochromatic transgene. (A) Organisation of an artificial insert at the RL5 locus in a murine erytholeukemia (MEL) cell clone. Construct consists of yeast centromere region from chromosome 6, a 10 kb array of human alpha satellite, and a GFP transgene with a human β-globin promoter and enhancer hypersensitivity sites. (B-C) Histograms showing GFP fluorescence of cells on the x-axis. (B) Basal GFP expression level of α-MEL cells (C) GFP expression in α-MEL cells after 1 µM TSA treatment for 48 hours.

The inserted P212 cassette encodes an enhanced GFP gene under a human β -globin promoter and a series of hypersensitivity sites 2, 3, and 4 (HS2, -3, -4) from the human β -globin locus control region (LCR) was inserted at the RL5 locus (Figure 3.1). Activation via HS2 is mediated by the histone acetyltransferase CREB binding protein (CBP) (Johnson, Norton and Bresnick, 2002) whilst HS3 is the binding site for the required transcription factors GATA-1 and Oct1 (Molete et al., 2002). HS4 in contrast acts as a terminal differentiation insulator of HS2 from the β-globin promoter, mediated by CTCF (Zhao et al., 2006). The β-globin promoter, when interacting with HS2, facilitates chromatin remodelling via SWI/SNF
complex HLTF, activating globin transcription (Mahajan and Weissman, 2002). By varying the artificial insert, Feng et al, (2005) demonstrated that eGFP with hypersensitivity sites 2 and 3 is permissive of both active and silenced states. Kim et al, (2009) showed that the basic eGFP cassette with hypersensitive sites, when inserted at the RL5 locus stably expressed eGFP for longer than 6 months without selection. This demonstrated a stably accessible chromatin environment at the RL5 locus within this cell line.

A modified p212 cassette, with a flanking YAC/BAC centromeric element from yeast chromosome 6, Kim (2009), silenced transgene expression (Chen et al., 2004; Suzuki, Kasai and Saeki, 2006). This cassette was further modified by inserting a 10 kb array of 11 monomer higher order α-satellite repeats from the human centromere of chromosome 21 (Figure 3.1A). This pYB-α insert showed the strongest silencing that Kim, (2009) tested and was decorated with H3K9me3 and H3K27me3, marks present at repressive pericentric heterochromatin (Peters et al., 2003). The expression of eGFP within pYB-α MEL cells was therefore considered to be a good system to identify compounds, which disrupt heterochromatic silencing and could give a clear phenotypic output.

To analyse basal eGFP expression in pYB-α cells, GFP levels were analysed by flow cytometry. The cells had a normal distribution, with only a few cells with elevated eGFP fluorescence (Figure 3.1B). To test the effect of changes in histone acetylation cells were treated with 1 μM Trichostatin A, an inhibitor of histone deacetylases, for 48 hours. The majority of cells maintained an elevated eGFP fluorescence however the distribution was biphasic, suggesting some heterogeneity within the cell population, which was consistent over time and treatments (Figure 3.1C). Flow cytometry
gating for GFP expression was defined by using MEL RL5 cells that lacked any eGFP transgene. The level of eGFP fluorescence within this reporter system is influenced by the underlying eGFP transgene transcription, and the amount of time after treatment that is required to change gene expression. Consequently, genes essential for heterochromatin formation after mitosis would take longer to activate eGFP transcription and have lower eGFP fluorescence. Therefore, for the purpose of this screen, a significant change in eGFP fluorescence after drug treatment was considered as a potentially relevant compound.

**Figure 3.2. Optimisation of eGFP transgene reactivation in MEL cells.** Graph showing fold change in GFP expression in α-MEL cells, containing the heterochromatic construct after drug treatment measured by flow cytometry. P212 untreated cells (left) lack the yeast centromeric sequences and α-satellite repeats, and GFP is constitutively expressed. RL5 untransfected loci (right) lack the eGFP construct entirely providing a negative control for background fluorescence. Fluorescence was normalised to α-MEL cells DMSO treated controls. Six compounds; 5′ Azacytidine, Aspirin, UNC0638, JNK II and Akt IV, were tested in α-MEL cells at 1 µM for 72 hours.
To investigate the reproducibility of this assay, 6 compounds (1 μM for 48h) were screened and compared to the expression level of the p212 cassette lacking any centromeric sequences; the RL5 cell line lacking any eGFP transgene was used as a negative control (Figure 3.2), and all samples were normalised to the 0.01% DMSO control samples. Canonical inhibitors of gene silencing, TSA and 5′ azacytidine (5′Aza) altered eGFP expression. Expression was more than the p212 eGFP expression suggesting that the effect goes beyond the repressive properties of the pYB-α-satellite insert, promoting gene activation.

Cells were also treated with 1 μM aspirin and UNC638. Aspirin, while still poorly understood, is known to inhibit NF-kB which has been shown to act as a transcriptional activator at the β-globin locus in erythroleukaemia cells (Moitreyee et al, 1998). As aspirin did not affect eGFP expression this suggests that NF-kB does not influence the human β-globin promoter in this repressed state, and suggests that the repressed state of this transgene prevents transcription factor binding. UNC0638 also did not affect eGFP expression. UNC0638 is a highly specific inhibitor of G9a/GLP methyltransferases responsible for H3K9-mono and -dimethylation. G9a/GLP function has been associated with selective looping between foetal and adult globin genes (Chaturvedi et al., 2009; Deng et al., 2013) and UNC0638 G9a/GLP inhibition has previously been used to facilitate looping to foetal globin promoters reducing β-globin expression (Krivega et al., 2015). Therefore, UNC0638 would be expected to inhibit eGFP expression if any looping occurred between the LCR hypersensitive sites and the human β-globin promoter. As UNC0638 had no effect on eGFP expression, chromatin looping is unlikely to function within this repressed construct. Investigating the signalling pathways associated with β-globin expression is also
important; JNK kinase activation regulates haemopoietic transcription factor degradation (Lee et al., 2010). Therefore, JNK2 inhibition should increase TF availability for gene activation. Limited re-activation of eGFP after JNK II inhibition suggests it is not involved in maintaining the repressive chromatin environment at the eGFP transgene. AKT also negatively regulates PLC kinase mediated β-globin expression (Follo et al., 2008, 2012). The lack of re-expression of eGFP after AKT inhibition provides further evidence that this model of gene silencing is not primarily mediated by transcription factors.
Figure 3.3. RFP and heterochromatic eGFP expression dynamics. α-MEL cells with a randomly inserted RFP lentiviral construct (Sigma. Cat No. SHC012V). Flow cytometry plots showing GFP on the y-axis and RFP fluorescence on the x-axis. (A-B) Silenced lentiviral RFP α-MEL clone, treated with (A) DMSO (B) 1µM TSA for 72 hours. (C-D) Expressed RFP α-MEL clone, treated with (C) DMSO (D) Chaetocin 1 µM for 72 hours (E) 10x images of RFP expressing α-MEL cells after 72 hours with DMSO or JQ1™ treatment showing GFP and RFP fluorescence.
To control for compounds, which generally affected gene expression, from heterochromatic modulators, and to improve this model system, a lentiviral system was used to insert an RFP gene under a CMV promoter across the genome (Sigma, Cat No. SHC012V). The random integration of this construct resulted in MEL clones that varied in eGFP and RFP fluorescence. Clones were selected that maintained a silenced eGFP with a silent or expressed RFP construct (Figure. 3.3A, C). When eGFP was re-expressed in the silenced RFP clone with TSA (1 \( \mu M \) 24 hours) there was no increase in RFP indicating that the transgene is silenced (Figure. 3.3B). This effect was also demonstrated in RFP expressing clones (Figure. 3.3D). However, RFP expressing clones did not see similar levels of eGFP activation but cells with higher RFP levels also had higher eGFP levels, limiting the sensitivity of the primary reporter. Therefore, for the initial FACS screen MEL clones with the silenced RFP construct were used, and validated using the RFP expressing clone. Of the compounds that significantly activated eGFP expression in Table 3.1 only Cdk inhibitor CR8 and Nicotinamide, which is thought to function in DNA methylation, affected RFP expression. When selected hit compounds identified using the RFP expressing clone were validated by live cell imaging, there was no significant change in RFP pixel intensity compared to the increase in eGFP fluorescence (Figure. 3.3E). This suggests that the compounds affect heterochromatic silencing, rather than regulators of transcription.
3.2 Screening for heterochromatic modulators

In the primary screen, over 250 compounds were tested with known and unknown targets (Appendix 1). Each compound was tested at 1 \( \mu \text{M} \) for 24 hours and compared to a DMSO control. 30 compounds showed significant and consistent eGFP expression (Table 3.1), and can be broadly grouped by target; HDAC, histone demethylase, SIRT-HDACs, signalling kinases, cell cycle, bromodomain, and unknown.

The most prominent of these was the HDAC group. This was not surprising as many HDAC inhibitors exist and are being investigated as therapeutics in a range of malignancies (Ceccacci and Minucci, 2016), and were prevalent in our library. Surprisingly, the level of eGFP expression differed significantly between the compounds. M344, an HDAC3 inhibitor with an IC50 of 100 nM, was less effective than broad HDAC inhibitors (S)-HDAC-42 and CAY10433, which are active in the \( \mu \text{M} \) range. This could suggest that HDAC3 is less important for eGFP reactivation than HDAC1/6. This is perhaps not surprising, as HDAC3 is known to deacetylate H3K27 to enable subsequent methylation. H3K27me3 mediated silencing tends to be mutually exclusive from constitutive H3K9me3 silencing, as was shown to be enriched over the pYB-\(\alpha\) construct (Kim et al., 2009). This therefore suggests that inhibitors of polycomb mediated silencing, will be less effective than H3K9me3 linked mechanisms.
Table 3.1. α-MEL eGFP reactivation compound screen. eGFP expression after α-MEL cells were screened with each compound in turn for 48 hours, and fluorescence determined by flow cytometry. Table of compounds that by a Bonferroni corrected two tailed t-test significantly affected GFP fluorescence after treatment for 48 hours at 1 µM (unless stated otherwise). n = >3.

<table>
<thead>
<tr>
<th>Drug</th>
<th>P Value</th>
<th>Mean fold change in GFP expression</th>
<th>Target</th>
</tr>
</thead>
<tbody>
<tr>
<td>MS-275</td>
<td>0.004925982</td>
<td>18.63136934</td>
<td>HDAC 1</td>
</tr>
<tr>
<td>CAY10603</td>
<td>0.013633511</td>
<td>13.29898959</td>
<td>HDAC6</td>
</tr>
<tr>
<td>N-oxalylglycine</td>
<td>0.009706582</td>
<td>11.3134046</td>
<td>A-ketoglutarate analogue</td>
</tr>
<tr>
<td>JQ1 +/-</td>
<td>0.001075345</td>
<td>9.919194763</td>
<td>Brd2/3/4/t</td>
</tr>
<tr>
<td>JNK IX</td>
<td>0.012350805</td>
<td>8.203121959</td>
<td>JNK kinase 2/3</td>
</tr>
<tr>
<td>EGFR CAY</td>
<td>0.005197979</td>
<td>6.847546494</td>
<td>EGFR</td>
</tr>
<tr>
<td>Nicotinamide</td>
<td>0.0007832</td>
<td>6.766163463</td>
<td>DNA methylation</td>
</tr>
<tr>
<td>CR8</td>
<td>0.045432001</td>
<td>6.024891933</td>
<td>Cdk1/2/3/5/7/9</td>
</tr>
<tr>
<td>Gemcitabine</td>
<td>9.11794E-05</td>
<td>5.747655185</td>
<td>Cytidine analogue</td>
</tr>
<tr>
<td>PKC CAY</td>
<td>3.49894E-08</td>
<td>5.39596518</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>Aurora A I</td>
<td>0.000114125</td>
<td>5.306265754</td>
<td>Aurora kinase</td>
</tr>
<tr>
<td>S-HDAC-42</td>
<td>0.008520126</td>
<td>4.495340903</td>
<td>HDAC</td>
</tr>
<tr>
<td>Polo CAY</td>
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<td>4.443171944</td>
<td>Polo kinase</td>
</tr>
<tr>
<td>Tubastatin A</td>
<td>0.000425538</td>
<td>4.159496234</td>
<td>HDAC6</td>
</tr>
<tr>
<td>CAY10433</td>
<td>1.60398E-05</td>
<td>3.236466453</td>
<td>HDAC</td>
</tr>
<tr>
<td>IOX1</td>
<td>4.4078E-06</td>
<td>2.875600431</td>
<td>JmJC demethylase</td>
</tr>
<tr>
<td>Cy19</td>
<td>0.030144393</td>
<td>2.771185384</td>
<td>Unknown</td>
</tr>
<tr>
<td>Emi1 (10uM)</td>
<td>0.021528568</td>
<td>2.72710909</td>
<td>Unknown</td>
</tr>
<tr>
<td>Dg19</td>
<td>0.001608853</td>
<td>2.554656165</td>
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</tr>
<tr>
<td>Dg16</td>
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<td>2.405384254</td>
<td>Unknown</td>
</tr>
<tr>
<td>CAY10398</td>
<td>0.020074572</td>
<td>2.220820726</td>
<td>HDAC1</td>
</tr>
<tr>
<td>trans-Resveratrol</td>
<td>0.00085766</td>
<td>2.125363958</td>
<td>SIRT1 activator</td>
</tr>
<tr>
<td>AG-014699</td>
<td>9.02063E-09</td>
<td>1.958926292</td>
<td>PARP</td>
</tr>
<tr>
<td>JGB1741</td>
<td>0.000356006</td>
<td>1.40895944</td>
<td>SIRT1 - HDAC</td>
</tr>
<tr>
<td>Bromosporine</td>
<td>0.002356856</td>
<td>1.079811982</td>
<td>Bromodomain family</td>
</tr>
<tr>
<td>M 344</td>
<td>0.002270078</td>
<td>0.780450208</td>
<td>HDAC 3</td>
</tr>
<tr>
<td>Tenovin-1</td>
<td>0.00045872</td>
<td>0.624920819</td>
<td>SIRT1/2 HDAC</td>
</tr>
<tr>
<td>Capecitabine</td>
<td>0.006403966</td>
<td>0.563737847</td>
<td>DNA synthesis</td>
</tr>
<tr>
<td>Phthalazinone pyrazole</td>
<td>0.004715884</td>
<td>0.443206574</td>
<td>Aurora A kinase</td>
</tr>
<tr>
<td>SAHA</td>
<td>0.000341222</td>
<td>0.385392863</td>
<td>HDAC</td>
</tr>
</tbody>
</table>
The high level of eGFP expression as a result of MS-275, and CAY10603 treatment is likely due to their high selectivity, and that HDAC1/6 have been implicated in haematopoiesis and could have a greater role in regulating gene expression in an erythroleukaemia background. Furthermore, HDAC1 functions in the NuRD complex and inhibition could suppress HP1 deposition (Huang et al., 2013; Shearstone et al., 2016).

The role of class III HDACs, known as Sirtuins (SIRT), remains unresolved (Kozako et al., 2014). In the MEL system both SIRT inhibitors and activators promoted eGFP expression (Table 3.1), although trans-resveratrol is reported as a SIRT1 activator, the mechanism is not direct and could function differently in this artificial system. The reactivation by SIRT1 inhibition/activation is significant but is below the basal expression of the p212 cassette suggesting a relatively weak effect on heterochromatic silencing. SIRT1 canonically functions to deacetylate H4K16 and H3K9 allowing for H3K9me3 and facilitates linker histone H1 binding and heterochromatin formation (Vaquero et al., 2004).

JmjC demethylases have recently been implicated in heterochromatin formation and JARID2 can recruit PRC2 complexes although this normally occurs in non-cycling cells (Son et al., 2013). JmjC demethylases can remove all three methylation states giving them broad functionality, initially demonstrated at H3K36. They have subsequently been shown to directly demethylate pericentric H3K9me3 (Klose et al., 2006; Whetstine et al., 2006). For JmjC domain containing proteins to demethylate substrates they require cofactors Fe(II) and α-ketoglutarate (Tsukada et al., 2006). JmjC inhibitors such as N-oxalyglycine and IOX1, promote eGFP expression, and could function by competitively inhibiting α-ketoglutarate binding to Fe(II) within
the domain. As this catalytic domain is conserved across the family this means inhibitors are non-specific. It is therefore surprising that catalytic inhibition of a family of demethylases known to directly affect pericentric heterochromatin, should cause eGFP expression.

Poly(ADP-ribose) polymerases (PARP) are known to bind at active promoters and at heterochromatic regions including α-satellite sequences, however this paradoxical function in gene regulation is not understood. At promoters, PARP1 binding excludes linker H1 association but PARP1 loss only affects a subset of bound genes that are both up- and down-regulated (Krishnakumar et al., 2008). Heterochromatic PARP1 interacts with the Nucleolus repressor complex (NoRC) (Strohner et al., 2001) and recruitment of PARP1-NoRC is facilitated by nascent RNA which acts as a substrate for parylation (Mayer et al., 2006). Research has demonstrated that this is a transient event after the passage of a replication fork to facilitate heterochromatin formation in mid-to-late S phase (Guetg et al., 2012). As MEL cells were treated for 48 hours with each compound, this would normally allow multiple complete cell cycles to take place so PARP inhibition could inhibit heterochromatin reformation explaining eGFP activation.
Perturbation to the cell cycle also resulted in eGFP expression. Inhibition of cyclins by CR8 and nucleotide analogues such as gemcitabine and capecitabine significantly de-repress the RL5 locus. Feng, (2005) demonstrated that insertion of p212 cassette with silenced or expressed eGFP, replication timing shifts from late-to-early. The relationship of gene expression, replication timing and chromatin is unclear, but by altering the length of S-phase via Cdk inhibition or nucleotide analogues could affect the replication timing of the eGFP transgene which may in turn regulate its chromatin environment.

A broad range of kinase inhibitors were found to activate eGFP expression: JNK II/III, EGFR, PKC, Polo, and Aurora (Table 3.1). As mentioned above the JNK pathway is thought to be antagonistic to \( \beta \)-globin expression (Lee et al., 2010). JNK II inhibition in Figure 3.2, did not, however, reactivate the eGFP transgene. JNK IX inhibitor of JNK II/III however resulted in an 8-fold increase in eGFP fluorescence. JNK III is known to function as a stress induced pro-apoptotic pathway independent of JNKI/II transcriptional regulation (Yang et al., 1997). Stress related proteins such as heat shock factor 2-\( \beta \), are regulated by the JNK pathway and are involved in erythroid differentiation (Hietakangas et al., 2001). Although exactly how JNK functions in differentiation remains undefined, JNK3 is known only to be expressed in a tissue specific manner in the brain, heart and testes. The three JNK genes are spliced to form 10 JNK isoforms that regulate TF activity independently of each other (Gupta et al., 1996). More recently, JNK kinases have been shown to directly phosphorylate H3S10 in interphase (Tiwari et al., 2012) whilst the combined and adjacent marks H3K9me3 and H3S10p have been associated with modulating heterochromatin through differentiation (Ribeiro-Mason et al., 2012). The effect of JNK IX inhibition on eGFP
expression may provide an insight to heterochromatin regulation in the MEL background.

During mitosis, H3S10 phosphorylation occurs via Aurora B rather than JNK (Hirota et al., 2005). The function of mitotic H3S10p on chromatin condensation remains unclear, as its presence causes HP1 proteins to dissociate during mitotic compaction. Aurora kinase inhibition by Aurora-A, or Phthalazinone pyrazole, caused eGFP expression. When viewed in combination with JNK inhibitions effect on eGFP expression, this suggests that H3S10p is required for maintaining the transgene silencing, even if the mechanism is indirect.

EGFR, PKC and PLC inhibition triggered eGFP expression. EGFR is a membrane receptor that can stimulate multiple kinase pathways, including PI3K/Akt. Akt inhibition, downstream of EGFR, does not however induce expression of the eGFP transgene. This suggests that the PKC/PLC pathway of the EGFR response is modulating the transgene expression. PLC is directly activated by EGFR, and results in downstream PKC activation (Hofmann et al., 1999). PKC once activated is a potent stimulator of the Ras/Raf/MEK/ERK pathway (Schönwasser et al., 1998). ERK activation is required to maintain haematopoietic undifferentiated cell states demonstrating it as a key regulator within an erythroid setting (Chan, Gu and Neel, 2013). How ERK modulates differentiation is not fully understood, although it has been shown to regulate key haematopoietic transcription factors including c-myc (Chen and Sytkowski, 2001). Additionally, PKC has been implicated in heterochromatin modulation directly via interacting with transcriptional intermediary factor 1B (TIF1B). TIF1B regulates KAP1/TRIM28 chromatin remodelling complex recruitment to chromatin.
TIF1B is recruited to heterochromatin by directly binding to HP1 proteins, but this is abrogated by its phosphorylation at Ser473 by PKC (Chang et al., 2008). Inhibiting PKC therefore could directly result in the loss of heterochromatin condensation via KAP1/TRIM28. This is most likely the mechanism behind eGFP expression, as ERK inhibitors by contrast did not result in eGFP activation (Appendix 1).

Intriguingly, bromodomain inhibition by JQ1 also caused eGFP expression; the effect was specific as the inactive enantiomer of JQ1 did not change expression (Appendix 1). Additionally, the less selective bromosporine compound also activated the eGFP transgene. These results were surprising as bromo-extra terminal (BET) proteins inhibited by JQ1 are known transcription factors found at active enhancers (Chapuy, McKeown and Lin, 2013). The role of BET proteins in haematological malignancies has been widely studied with its function in oncogene activation attributed to activation at super enhancers (Lovén et al., 2013). Therefore, the role of BET proteins in regulating heterochromatic silencing differs from their canonical role.
3.3 BET mediated eGFP expression

To better understand the role of BET inhibitors MEL cells were treated with bromosporine for 48 hours at 0.05 \( \mu \) M to 1 \( \mu \) M, prior to analysis by FACS in the same method as described earlier (Figure. 3.4A). eGFP was upregulated after 0.5 \( \mu \) M treatment, and re-expression correlated with increased cell death. This suggests that the function of BET proteins in heterochromatin condensation is essential for cell viability.

![Figure 3.4. Bromosporine eGFP reactivation and cell cycle arrest. (A) MEL cells treated with increasing concentrations of pan BET inhibitor, bromosporine for 48 hours. GFP fluorescence and cell death was determined by flow cytometry. (n=3) (B) Stacked bar plot showing cell cycle analysis by high content imaging on ScanR platform. MEL cells were grown on gelatin coated dishes and treated with bromosporine at various doses for 48 hours prior to imaging. Error bars = SEM between biological replicates.](image-url)
As these cells are non-adherent, plates were coated with 0.1% gelatin, to facilitate cell adhesion and access to microscopy techniques. MEL cells were treated with 0.1 µM to 5 µM bromosporine for 48 hours, as this covered the reactivation of eGFP detected by FACS (Figure. 3.4A). The ScanR analysis platform was used to determine cell cycle stage of more than 500 cells per treatment. Bromosporine caused a dose dependent increase in G2/S phase after treatment (Figure. 3.4 B) which suggests that replication is impaired and cells are therefore not allowed to progress through mitosis.

To analyse the relationship between eGFP expression and cellular proliferation, cells were treated with a log-dose range of selective BET inhibitor I-BET151 (Figure 3.5). MEL cells were treated with I-BET151 for 48 hours before the addition of cell viability reagent PrestoBlue for 3 hours prior to measuring the fluorescence at 590-615 nm. Independent samples were normalised to a DMSO 0.01% control. 10-1000 nM I-BET151 gives an 80% reduction in cell viability (Figure 3.5 A) and 50-5000 nM I-BET151 affects eGFP levels measured by FACS (Figure 3.5 B). This suggests that the effect on cell viability precedes BET inhibitions effect on condensed heterochromatin.
Figure 3.5. Relationship between IBET151 eGFP reactivation and cell viability. α-MEL cells treated for 48 hours with a log-dose range of i-BET151. (A) Cell viability was determined using a Presto Blue™ assay (n=6). (B) Fold change in GFP fluorescence in response to IBET151 measured by flow cytometry.
As the previous experiments investigating eGFP involved 48-hour treatments, which affect multiple cell cycles, it seemed desirable to investigate the kinetics of eGFP expression post treatment. While coating wells in gelatin was sufficient for cell attachment before fixation for standard microscopy, this method still resulted in cellular detachment over time. This led to a change in the depth of field for individual cells preventing tracking over time. To reduce this movement, cells were seeded in matrigel (Hughes, Postovit and Lajoie, 2010). When incubated post seeding the matrigel polymerizes preventing the movement of the non-attached MEL cells and enabled imaging of wells at 10x over 84 hours to investigate the kinetics of eGFP expression. To investigate kinetics of eGFP expression a MEL clone expressing RFP was also used (Figure 3.3 C-E) which allowed a comparison of eGFP expression to general gene activation.

To exclude compound specific effects cells were treated with a half-log dose range of the three BET inhibitors, previously identified to cause eGFP expression: Bromosporine, JQ1\textsuperscript{+/-}, and I-BET151 (Figure 3.6). Cells were also treated with dose ranges of 5’Azacytidine and Chaetocin, which inhibit DNA methylation and H3K9me3 respectively. DNA methylation, while not affecting chromatin condensation, regulates endogenous \(\beta\) -globin transcription in MEL cells at the LCR and down-stream of the 3’ end (Yisraeli \textit{et al.}, 1988; Kotkow and Orkin, 1995).
For analysis, the change in RFP expression was subtracted from eGFP expression. RFP pixel intensity was however only affected after chaetocin treatment. This resulted in a decrease in eGFP/RFP expression ratio after low dose chaetocin (Figure 3.6 B). Intriguingly, the speed of eGFP activation differed between 5’Azacytidine and Chaetocin. Re-expression from chaetocin was first detected at 12 hours post treatment. 5’Azacytidine by contrast required 36 hours post treatment for activation of the eGFP transgene (Figure. 3.6 A-B). Also unexpectedly, low dose chaetocin resulted in increased silencing.

All three selective BET inhibitors: I-BET151, JQ1\(^+/-\), and Bromosporine caused eGFP expression 24 hours after treatment which sits in-between Chaetocin and 5’Azacytidine eGFP activation. Additionally, the pan BET inhibitor Bromosporine required a higher dose to achieve the same level of eGFP expression compared to JQ1\(^+/-\) and I-BET151 (Figure 3.6 C-E) with JQ1\(^+/-\) having a 2.5 fold greater effect than Bromosporine. I-BET151 was approximately 0.6 fold more effective at eGFP reactivation than JQ1\(^+/-\); as JQ1 and I-BET151 are known to be active at inhibiting BET proteins with the similar potency, this effect is most likely due to differences in selectivity within the BET family.
**Figure 3.6. Heterochromatic eGFP activation kinetics.** α-MEL cells embedded in Matrigel™ were imaged over 84 hours using an Incucyte microscope. GFP fluorescence is relative to DMSO controls at each time point. Fold change in GFP fluorescence is shown for cells treated with compounds 12 hours after seeding. (A) 5’Azacytidine (B) Chaetocin (C) I-BET151 (D) Bromosporine and (E) JQ1*. 

*Note: The asterisk (*) indicates a specific condition or treatment in the experiment.
To investigate the effect of BET inhibition on epigenetic marks, samples treated with BET inhibitors were analysed by reverse phase protein arrays. Protein lysates were spotted onto arrays in triplicate serial dilutions (Figure 3.7), the arrays were clamped into 12 chambers per array and incubated with primary and secondary antibodies. The $R^2$ value, used to determine how close data fits a line of regression, was measured for signal intensity across the duplicate spot serial dilution was checked as a quality control.
Figure 3.7. Reverse phase protein arrays. Protein array methodology. Lysates were spotted onto arrays in a four series dilution in duplicate. Primary and secondary antibodies were sequentially incubated in the chambers and imaged. Antibody fluorescence signal was quality controlled by analysing the R² of the serial dilution. Samples 1, 12, and 23 represent the H3K9 acetylation serial dilutions above, indicative of the sample spotting order (number shown on graph). All samples with R² value <0.95 were excluded.
33 histone modifications or chromatin binding proteins were assayed after 1 μM I-BET151 treatment for 6 hours and 24 hours. The 6 hour and 24 hour treated samples were collated to determine significant changes that were sustained over time (Figure. 3.8). The only significant changes after BET inhibition were elevated levels of unmodified and dimethylated H3K4. Although not significant (p=0.064), H3K4 monomethylation correlated with the elevated signals from H3K4me2 and unmodified antibodies. This correlation would be expected with loss of H3K4me3, a mark of active transcription and is not surprising given the role of BET proteins as transcription factors (Kyoo Jang et al., 2005; W. Zhang et al., 2012). As expected there were no significant change in global levels of H3K9 methylation states or BET proteins Brd2, and Brd3, but of interest were a near significant loss of H3K27me3 (p=0.059) with elevation of H3K27me2, and the general trend for a subtle increase in histone acetylation (H4K16ac, H4K12ac, H4K5ac). BET proteins are known to bind to acetylated H4, so a compensatory increase in H4 acetylation is possible (Filippakopoulos et al., 2012) although it remains unknown whether a general increase in histone acetylation would affect heterochromatin.
Figure 3.8. Global Epigenetic modification changes with IBET151. Boxplots showing changes in global epigenetic marks after BET inhibition in α-MEL cells, measured by reverse phase protein arrays. Cells treated with 1 µM IBET151 for 24 hours (n=9) and analyzed by Bonferroni corrected 2-way ANOVA to calculate significance.
The eGFP transgene is known to be marked with H3K9me3 (Kim et al., 2009). Therefore, the levels of H3K4me3, and H3K9me3 at the eGFP transgene were evaluated, given the global changes in H3K4 methylation detected by RPPA (Figure 3.8). Chromatin immunoprecipitation was used to analyse the effect of BET inhibition at the amylase, eGFP, murine β-globin, and major satellite (Figure 3.9). Amylase is constitutively repressed throughout normal haematological differentiation, whilst a subset of haematological tumours have ectopic amylase expression independent of genetic translocations (Weiss, Edmondson and Wertman, 1951; Ohtsuki et al., 1989). However, amylase is repressed in MEL cells, providing a locus of potential facultative heterochromatin (Kim et al., 2009). The endogenous β-globin gene is in a poised state in undifferentiated MEL cells and undergoes chromatin decondensation and expression upon differentiation (Smith and Yus, 1984) providing a poised silent loci in these MEL cells. Finally, major satellite repeats are pericentromeric and are packaged as constitutive heterochromatin.

Both JQ1⁺/⁻ and I-BET151 treatment caused a large reduction in H3K9me3 over the eGFP transgene (Figure 3.9 B). There was also an enrichment of H3K4me3 compared to control. This change in histone modifications correlates well with gene activation, although treatment with BET inhibitors did not consistently increase Brd4 occupancy at the transgene. Treatment of BET inhibitors decreased H3K9me3 and H3K4me3 at the poised β-globin gene (Figure 3.9 C) and Brd4 occupancy was also reduced especially after IBET151 treatment.

Control facultative and constitutive loci, amylase and major satellite showed reductions in H3K9me3 after BET inhibition which was exacerbated after
IBET151 treatment (Figure 3.9 A,D). Unlike at the eGFP transgene there was no elevation of H3K4me3 at amylase or Major satellite, nor a consistent increase in Brd4 after BET inhibition.

Figure 3.9. Chromatin immunoprecipitation for H3K9me3, H3K4me3, and Brd4 after BET inhibition. MEL cells were treated for 24 hours with DMSO, JQ1“+”, or IBET151. qPCR’s were performed at four loci and compared to ChIP input samples (A) Amylase (B) GFP transgene (C) Murine β-globin and (D) Major Satellite.
3.4 BET inhibition affects cell signalling

Cellular epigenetic profiles represent the programmed state, and their adaption to the cellular environment (Ravelli et al., 1998; Borengasser et al., 2013). Inhibiting an epigenetic process such as acetyl-lysine binding is therefore likely to alter a cell’s response mechanism. Either compensatory changes to signalling pathways could occur to overcome a BET protein reduction or cellular adaption to signalling will be altered. To investigate if BET inhibitors affected cellular signalling, MEL cells were treated with JQ1+/− or I-BET151 1 µM for 24 hours. Cell lysates were prepared and spotted onto Zeptozen™ arrays for 87 antibodies. JQ1+/− and I-BET151 samples were collated to reduce off target drug specific effects and compared to controls (Figure 3.10, Appendix 2). Significant consistent changes with BET inhibition are summarised in Table 3.2.
Figure 3.10. Signaling kinase changes after BET inhibition. Boxplots of kinase proteins and their phosphorylated states after BET inhibition in α-MEL cells. Treatments with 1 µM JQ1*/- or IBET151 for 24 hours were collated together to remove off target compound specific effects. 2-way Bonferroni was performed to determine significance.
As one might expect BET inhibition resulted in a decrease in Brd4 protein levels, whilst changes in kinases suggest that BET inhibition and loss of Brd4 adversely affects canonical EGFR signalling.

The canonical EGFR pathway is Ras/Raf/MEK/ERK, which up regulates multiple transcription factors including c-myc and Elk-1. Importantly, the downstream component ERK1/2 shows a significant loss in phosphorylation after BET inhibition (Table 3.2). MEK is upstream of ERK and shows a significant increase in phosphorylation after BET inhibition indicating the cell is stimulating ERK signalling. Hyper-phosphorylation of ERK signalling is a known resistance mechanism to loss of ERK signalling via receptor tyrosine kinase (RTK) or Ras/RAF/MEK inhibition.

BRAF can be alternatively activated by PKA or PKC independently of Ras, both of which are phosphorylated after BET inhibition. Both these pathways are stimulated by metabolic compounds cAMP and PIP\textsuperscript{2} respectively. Increased cAMP stimulates PKA mediated Rap1 phosphorylation both of which are up-regulated by BET inhibition. Rap1 can then directly activate BRAF independently of Ras and is known to regulate multiple haematological response pathways (Katagiri \textit{et al.}, 2002). PKC can also directly activate BRAF. PKC activity is modulated by PLC that requires the PIP\textsuperscript{2} metabolite. PIP\textsuperscript{2} also acts as the substrate for PI3K signalling, which is also stimulated by BET inhibition and activation of the PI3K/Akt pathway, and is a well characterised mechanism to mediate loss of ERK signalling (Vergani \textit{et al.}, 2011; Cante-Barrett \textit{et al.}, 2016).
Table 3.2. Significant kinase changes to BET inhibition. List of two-tailed t-test significant changes to kinase signaling in α-MEL cells. α-MEL cells were treated with 1 µM I-BET151 or JQ1 for 24 hours and the signaling effects binned together and normalised from DMSO controls. Bonferroni corrected ANOVA (*, p < 0.05) (**, p < 0.01).

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Δ from DMSO (%)</th>
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</tr>
</thead>
<tbody>
<tr>
<td>MAPKAPK-2</td>
<td>91.92744</td>
<td>3.82085E-9 **</td>
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<tr>
<td>PKC-zeta/lambda P Thr410/403</td>
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<td>1.4041E-8 **</td>
</tr>
<tr>
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<td>3.4141E-6 **</td>
</tr>
<tr>
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<td>68.94439</td>
<td>6.83351E-6 **</td>
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<td>S6 Ribosomal Protein</td>
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<td>0.00026 **</td>
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<tr>
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<tr>
<td>FRA1 (R20)</td>
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PIP² is also known to modulate the targeting of Src (Corte, Gettemans and Vandekerckhove, 1997; Benesch et al., 2002) which in turn phosphorylates Stat3 resulting in increased invasion and poor prognosis in lung cancer (Carretero et al., 2010). Src upstream receptor Met has significantly reduced phosphorylation after BET inhibition whereas, its downstream effector Stat3 protein level is up-regulated. LKB1, which normally inhibits Src activation, is known to be supressed by elevated cAMP and is reduced by BET inhibition. This suggest that normal up-regulation of Src signalling after loss of EGFR is also prevented upon BET inhibition (Mueller et al., 2008) and a recent study implicated Brd2 as a regulator of Stat signalling hinting at a potential mechanism (Liu et al., 2014).

Stress activated MAPK signalling (SAPK/JNK) is also up-regulated after BET inhibition (Table 3.2) and the SAPK/JNK downstream partner c-Jun is more phosphorylated. SAPK/JNK when activated normally functions to activate p53 dependent cell arrest but after oncogenesis is known to mediate mesenchymal endothelial transition (MET), and pluripotency (Schramek et al., 2011; Neganova et al., 2016). One mechanism functioning in these pathways is the stabilisation of c-myc preventing its degradation after SAPK/JNK activation (Alarcon-vargas and Ronai, 2004). The modulation of ERK/Stat3/PI3K/JNK pathways after BET inhibition suggests that BET proteins function in regulating ERK mediated c-myc transcription, for which Brd4 is well known (Lovén et al., 2013).
3.5 Discussion

In the compound screens undertaken here multiple targets were identified as modulating heterochromatic silencing of an eGFP transgene. This synthetic model system generated by the Larionov lab, was previously used to investigate the ability of the underlying genetic sequence in modulating heterochromatin formation and gene silencing (Ebersole et al., 2005; Kim et al., 2009). This system is clearly regulated by the HDAC inhibitor TSA, and DNA methylation inhibitor 5’Azacytidine (Figure 3.2). TSA treatment both elevates histone acetylation and gene activation and has been shown to disrupt heterochromatin formation by mediating loss of H3K9me3 and HP1 proteins (Taddei et al., 2001). Loss of DNA methylation does not normally lead to chromatin de-condensation (Gilbert, Thomson, Boyle, Allan, Ramsahoye and Wendy A Bickmore, 2007) but DNA methylation is known, however, to regulate β-globin expression, and the eGFP transgene is under a human β-globin promoter. Given that MEL cells are of an undifferentiated haematological background, the β-globin promoter is normally poised (Figure. 3.9 C). Upon differentiation α- and β- globin genes are up-regulated in these MEL cells (Friend et al., 1971) suggesting that differentiation factors may also influence eGFP expression (Figure. 3.9 C).

The positive results in Table. 3.1, further validated this model, as multiple inhibitors of heterochromatic factors resulted in eGFP activation (HDAC, JmjC demethylases, PARP). An increase in eGFP expression by BET inhibition (JQ1+ and Bromosporine) is surprising as BET proteins are universally accepted as transcriptional activators across cell types and lineages (Korb et al., 2015; Roe et al., 2015; Najafova et al., 2017). As BET
proteins bind to acetylated lysines, this contradicts the role of CBP mediated acetylation at HS2 to activate the β-globin promoter (Johnson, Norton and Bresnick, 2002).

As demonstrated in figures 3.4 A and 3.5, eGFP activation by BET inhibition correlates with cell death. As these cells are stressed and BET protein Brd4 is known to stay bound to chromatin throughout mitosis, cell cycle stalling in Figure 3.4 B is expected (Zhao et al., 2011), however a correlation with cell death is not necessarily causal.

To investigate the kinetics of eGFP activation, MEL cells were embedded in Matrigel with the ratio of eGFP to RFP pixel intensity measured by live cell imaging (Figure 3.3 E). BET inhibition, resulted in decreased confluence as expected from Figure 3.4 B, whilst RFP pixel intensity remained constant. 5’Azacytidine treatment required multiple cell cycles to give eGFP expression (Figure 3.6 A) which is in line with DNA methylation in haematopoiesis causing globin gene expression (Lessard et al., 2015). Chaetocin, an inhibitor of Suv39 methyltransferases, was used as a drug to directly alter heterochromatin (Figure 3.6 B). Chaetocin did promote eGFP expression in the primary screen, but was toxic causing high levels of cell death. The further suppression of eGFP by low-level chaetocin treatment was due to an increase in RFP expression, which affected the GFP/RFP ratio, however eGFP activation was detected after 12 hours treatment, indicating loss of heterochromatin repression.
BET inhibition caused dose dependent eGFP expression 24 hours after treatment using a pan inhibitor bromosporine (Figure 3.6 D) and selective inhibitors JQ1+/− and I-BET151 (Figure 3.6 C, E). Bromosporine was the least effective, followed by I-BET151 and JQ1+/−. As a pan BET inhibitor bromosporine binds to proteins including CECR2, CBP and EP300 in addition to Brd- proteins (Picaud et al., 2016). As CBP mediated histone acetylation at HS2 greatly enhances normal β-globin expression, eGFP expression after inhibition of these proteins is intriguing (Johnson, Norton and Bresnick, 2002). eGFP expression from selective inhibitors JQ1+/− and I-BET151 would suggest that eGFP activation is independent of pan BET inhibition, and that this process is dominant over CBP-EP300 mediated acetylation. As CBP acetylation and GATA-1 recruitment is required for differentiation dependent globin expression, this suggests that Brd- protein loss directly causes heterochromatin loss (Cho et al., 2008). BET specific inhibition, while causing eGFP expression to a greater level than chaetocin treatment, was only detected 24 hours post treatment. The loss of heterochromatin therefore could require replication. This supports work from the Spector lab, where artificial tethering of Brd4 to heterochromatin did not cause HP1 loss and gene activation, but accelerates post mitotic de-compaction (Zhao et al., 2011).

The only histone modifications globally affected by BET inhibition were H3K4 unmodified, mono- and di-methylated (Figure 3.8) whilst at the eGFP transgene H3K4me3 is enriched (Figure 3.9 B). Together this data infers a loss of H3K4 acetylation, which is normally found upstream of active promoters (Wang et al., 2008). Promotion of H3K4me2 –me3 over H3K4 acetylation is mediated via the Set-1 containing complex (COMPASS), although the explicit function of H3K4 acetylation is poorly understood.
(Guillemette et al., 2011). This may be functional however as the tandem bromodomains (BD1 and BD2) in Brd- proteins have different specificities (Wu and Chiang, 2007). Although highly conserved, the two bromodomains only share ~44% homology and have been demonstrated to form homodimers (BD1-BD1, BD2-BD2) in solution (Nakamura et al., 2007). This has also been inferred in Brd4 where BD1 preferentially binds to the di-acetylated H4K5acK8ac motif; in contrast Brd4 BD2 preferentially binds to H3K4acK9ac over single H3K4 acetylation (Filippakopoulos et al., 2012). This indicates that BET protein binding to H3 or H4 is mediated by different bromodomains, where H3 needs to be acetylated. If BD2 binding to H3K4 acetylation is inhibited this would increase the exposure of the modification to turnover by COMPASS further preventing Brd- protein binding. However, H3K4 acetylation is associated with gene activation and as such would not be expected to affect heterochromatic regions.

ChIP-qPCR data shows BET inhibition causes a loss of H3K9me3, and enrichment of H3K4me3 at the eGFP transgene (Figure 3.9 B) whilst endogenous facultative and constitutive heterochromatin loci, amylase and Major satellite, respectively, show moderate loss of H3K9me3 but no enrichment of H3K4me3 (Figure 3.9 A, D). This suggests that the BET proteins role in heterochromatin maintenance is not limited to the artificial transgene, and appears to be a universal property of heterochromatin, even without gene activation. At a poised promoter, such as the endogenous β-globin, there is no significant change in H3K9me3/H3K4me3 ratio suggesting that this locus remains poised, although both marks are reduced (Figure 3.9 C) suggesting that BET inhibition does not induce erythroid differentiation within this system.
Aside from differentiation, BET proteins have been implicated in multiple signalling cascades. Brd4 interacts with the activated NF-kB subunit RelA and is required for its activation in the inflammation response (Huang et al., 2009). Brd4 can also act to insulate loci from signalling process such as DNA damage processes by recruiting condensin II complexes maintaining silencing (Floyd et al., 2013).

c-myc is a master regulator of haematopoiesis and its expression is regulated by Brd4. At the murine erythroid developmental stage, Myc is normally supressed by GATA-1 (Rylski et al., 2003) but MEL cell proliferation is driven by aberrant up-regulation of c-myc that is lost upon differentiation (Lachman and Skoultchi, 1984; Coppola and Cole, 1986).

To elucidate the effect of BET inhibitors on kinases, reverse phase protein arrays were used to analyse multiple signalling pathways in parallel (Figure 3.10). BET inhibition leads to increased Brd4 turnover, while Brd2 and Brd3 were previously shown to be unaffected (Figure 3.8). This would suggest that Brd4 mediates the effect of BET inhibition on heterochromatin.
Figure 3.11 MAPK signaling pathways. Diagram showing signaling pathways affected by 1 µM BET inhibition, for 24 hours. Canonical MAPK signaling pathway (right side) stabilises c-Myc. Compensatory PI3K/Akt signaling (center) can also lead to ERK mediated c-Myc stabilisation, which can also increase c-Myc expression. JNK/SRC signaling (left) associated with loss of cell adhesion factors and EMT-like transitions involving c-Myc stabilisation.
BET inhibition also caused significant loss of MAPK signalling from membrane receptors (Met) to downstream ERK (table 3.2). MEK and PKA, components upstream of ERK have increased protein levels but phosphorylation levels are not significantly altered. Protein level increase would remove their abundance as a factor regulating MAPK signalling, a logical response to loss of phosphorylation. Upon BET inhibition, alternative signalling pathways I3K and PKC are hyper-phosphorylated. This suggests that PIP2 signalling has been up regulated. PKC can act as an alternative method for ERK activation, while PI3K pathway is an alternative method of stimulating c-myc expression (Chen and Sytkowski, 2001). The stimulation of these pathways in addition to STAT and SAPK signalling suggested in figure 3.11, indicates that BET inhibition leads to a loss of MAPK signalling, for which the cell compensates through alternative pathways. Intriguingly, inhibitors of these pathways (SAPK/JNK, EGFR/MAPK, PKC) also lead to eGFP expression in the preliminary screen.
To conclude the MEL eGFP system proved to be a robust synthetic system of heterochromatin. Multiple signalling kinases and BET inhibitors, in addition to classical heterochromatic mechanisms, were identified as inducing eGFP expression. The effect of BET inhibition was not compound specific, suggesting it was not due to off-target effects. eGFP activation correlated with G2 arrest and cell death. Heterochromatic marks were also lost from endogenous facultative and constitutive heterochromatic loci. The loss of H3K9me3 at the eGFP transgene was accentuated compared to the endogenous loci, likely due to the gene transcription, for which active marks were not found at the endogenous loci. There was a global increase in H3K4 methylation by BET inhibition suggesting a global increase in transcription, which suggests an alternative role from transcriptional activation for BET proteins.

This is supported by recent work from the Escaffit lab, that recently demonstrated Brd2 binding to pericentric heterochromatin (Grézy et al., 2016). They demonstrated that TIP60 histone acetyltransferase deposits H4K12ac that acts as a binding site for Brd2 and depletion of TIP60 or Brd2 leads to pericentric satellite transcription, conducive to a de-condensed chromatin structure. The work presented here in MEL cells suggests that Brd4 has a role as it is the only Brd- protein depleted, although this requires further investigation to rule out Brd2. Loss of Brd4 has also been shown to globally increase accessibility to nucleases indicative of a de-condensed chromatin fibre, although not specifically demonstrated at heterochromatin loci (Wang et al., 2012a). Together this suggests BET proteins function at silenced loci in addition to their role in transcriptional activation.
Chapter 4: Epigenetic factors in tumor drug resistance and invasion

4.1 Introduction

The previous work in MEL cells identified MAPK signalling as a method of modulating heterochromatin structure (Chapter 3) but aberrant MAPK signalling is also a hallmark of oncogenesis. Hahn and Weinberg (2002) first modelled human cells in vitro proposing six essential steps to oncogenesis: independence from proliferative and anti-growth extracellular signals, freedom from replication limitation, apoptotic/senescence evasion, and gaining invasion and angiogenesis abilities. Through human intervention, acquiring drug resistance can also be considered an essential trait of tumour development. MAPK signalling cascades have been associated with each of these processes, and their mis-regulation is a common occurrence in a tumour setting, as suggested by Downward, (2003).

ERKs upstream effectors Ras and Raf have high mutational frequency in multiple malignancies, with Ras mutations found in 20-30% of all human tumours (Bos, 1989). These are invariably found in protein regions involved with GTP hydrolysis, causing constitutive activation. Importantly, this information has led to the development of multiple highly specific Ras/Raf/MEK inhibitors, to prevent ERK driven tumour survival. These can provide a significant benefit to melanoma patients where 45% present with BRAF mutations whilst another 20% harbour NRAS mutations (Hocker and
Tsao, 2007; Flaherty et al., 2012), but unfortunately, ERK driven tumours rapidly develop resistance leading to less than five months increased survival. In ~82% of BRAF V600E patients, resistance to BRAF/MEK inhibition is linked to reactivation of MAPK, PI3K and ERK signalling (Long et al., 2014). Of these V600E BRAF patients amplification only occurs in 20% of MEK resistant cases, whereas hyper-phosphorylation induced by V600E BRAF overexpression is in 80% of cases (Shi et al., 2012). Therefore the largest share of acquired resistance to MEK inhibition is not due to de novo genetic mutation, rather it is through an epigenetic response.

Many transcriptional regulators have been implicated in MEK inhibition (MEKi) acquired resistance. PTEN, a negative regulator of PI3K signalling is lost upon MEKi, while MITF (a PI3K activator) is upregulated (Wee et al., 2009; Ferguson et al., 2017). Additionally Ras activator ERBB3 is also upregulated leading to Ras hyper-phosphorylation (C. Sun et al., 2014).

Recent work has demonstrated that acquired resistance to ERK signalling inhibition correlates with large scale alterations in genome regulation similar to epithelial mesenchymal transition (EMT) (Jakobsen et al., 2016; Byeon et al., 2017). This leads to a more invasive tumour type and poor patient survival. Altered polycomb silencing has been implicated in an EMT-like transition in oestrogen receptor positive breast carcinoma background, demonstrating epigenetic processes within EMT (Javaid et al., 2013). Understanding how MAPK signalling influences genome regulation inducing an EMT-like transition is therefore fundamental to understanding how ERK driven tumours acquire resistance.
4.2 Development of acquired resistance

MEK inhibition model

Trametinib is a highly selective MEK inhibitor, that prevents ERK phosphorylation in a dose dependent manner <100 nM in HCT116 cells (Abe et al., 2011). In order to identify epigenetic mechanisms important for MEKi acquired resistance, HCT116 cells were treated with trametinib in increasing doses up to 100 nM over three months. HCT116 cells that could survive in 100 nM trametinib were considered to have overcome MEKi. Invasion assays (John Dawson, Neil Carragher lab) demonstrated that trametinib resistant cells had altered cellular morphology, decreased cell-to-cell contact, and increased motility (Figure 4.1 A). Trametinib treatment in sensitive HCT116 cells caused a decreased thickness of the cell layer in addition to reduced haematoxylin and eosin (H&E) staining, indicative of reduced cell density (Figure 4.1, A). HCT116 cells resistant to MEKi (TRAMR) maintain matrix invasion and cell layer thickness after trametinib treatment and H&E staining was slightly reduced. The decreased cell-to-cell contact and increased motility mimics the patient phenotype of MEKi acquired resistance due to an EMT-like transition (Byeon et al., 2017). TRAMR cells had increased survival at less than 100 nM trametinib treatment but similar cell survival to the sensitive HCT116 cells at concentrations about 100 nM (Figure 4.1 B) suggesting that resistance is acquired by hyper-activation of the Ras/Raf/MEK/ERK pathway. This was sustained even after the removal of trametinib stimulation for two weeks, demonstrating a new stable cellular state.
Figure 4.1. Development of Trametinib resistance. (A) Organotypic invasion assay (carried out by John Dawson). HCT116 or TRAMR cells were cultured on fibroblast-collagen matrices for 7 days with DMSO or 5 nM Trametinib. Images are H&E stained cross sections. (B) Cell viability data after trametinib treatment in HCT116 or TRAMR cells. Cells were treated with a log-dose of trametinib with viability determined by Presto Blue reagent after 48 hours and imaged on a Victor3 multi-label plate reader at 540/590 nm.
HCT116 and TRAMR cells were treated with 300 nM trametinib for 24 hour. Cell extracts were prepared by John Dawson, spotted onto reverse phase protein (RPPA) arrays and analysed using 185 antibodies against multiple signalling pathways. All the experimental data was collected and analysed (Appendix 3). Significant changes between samples are listed in table 4.1. The only significant change between DMSO treated HCT116 and TRAMR cells was ERK1/2 phosphorylation, which was upregulated in TRAMR cells (2.43 fold increase, p = 0.015), an expected result for a resistance mechanism linked to ERK hyper-phosphorylation.

Table 4.1. Significant kinase signaling changes in response to trametinib.

<table>
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<tr>
<th>Antibody</th>
<th>Basal Δ TRAMR p-value</th>
<th>HCT116 Δ Trametinib p-value</th>
<th>TRAMR Δ Trametinib p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>ErbB-2/Her2/EGFR P Tyr1248/Tyr1173 Met P Tyr1234 PLC-gamma1 P Tyr783 SHP2 P Tyr542 FLT3 P Tyr591</td>
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<td>3.79 &lt;0.001</td>
<td>2.09 0.021</td>
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<td>p44/42 MAPK (ERK1/2) P Thr202/Thr185, Tyr204/Tyr187 SAPK/JNK P Thr183,Tyr185 p53 P Ser15</td>
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<td></td>
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<td></td>
<td>0.93 0.906</td>
<td>1.40 0.564</td>
<td>1.97 0.041</td>
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</table>
Similar to previous studies, trametinib treatment in both cell lines triggered upregulation of ERBBs activation (C. Sun et al., 2014). Significant increases in phosphorylation after trametinib treatment were also found in HCT116 cells for: Met and FLT3 receptors, downstream PLC-gamma and SHP2, and SAPK/JNK (table 4.1). Met and FLT3 cell membrane receptors can activate PI3K signalling via PLC-gamma and SHP2 and are known to be upregulated in resistance to MAPK inhibition increasing cell motility (Usatyuk et al., 2014; Nogami et al., 2015). SAPK/JNK stimulation has also been implicated in EMT transition by inhibiting EZH2 mediated polycomb gene silencing, and is further evidence that trametinib treatment in this in vitro system is a good model for trametinib response (Li et al., 2016). While these proteins are also upregulated in TRAMR cells after trametinib treatment, only Met phosphorylation is significant. Additionally p53 Ser15 phosphorylation is also upregulated after trametinib treatment in both cell lines, indicating p53 mediated transcription (Loughery et al., 2017). Together these data suggest that trametinib treatment in HCT116 and TRAMR cells provides a useful model for analysing before and after acquired drug resistance in an EMT-like manner.
4.3 Identifying epigenetic components of acquired trametinib resistance

To identify mechanisms involved in acquired MEKi resistance, compounds were screened in a dose matrix with MEK inhibitor trametinib (Figure 4.2). If the compounds are synergistic then the combination of both drugs will increase cell death when compared to each compound singularly. Identifying differences in responses between HCT116 and TRAMR cells can be used to infer that the compound’s target protein is implicated in acquiring trametinib resistance. 42 selective inhibitors were screened in a dose matrix across 21 target classes identified as regulating synthetic heterochromatin in Table 3.1, in combination with trametinib. These included selective inhibitors of heterochromatin components, BET inhibitors and the kinase inhibitors identified in the MEL screen as modulating heterochromatin structure. Eight compounds were identified, as having combinatorial effects with trametinib or altered effects between HCT116 and TRAMR cells (Figures 4.3, 4.4). Two kinase inhibitors demonstrated altered profiles in TRAMR cells compared to HCT116. One of them, GW843682X is a selective inhibitor of PLK kinase (Lansing et al., 2007). In normal HCT116 cells PLK inhibition demonstrates no combinatorial effect with trametinib and limited efficacy singularly (Figure 4.3 A). TRAMR cells demonstrate increased sensitivity to PLK inhibition singularly (Figure 4.3 B). However, GW843682X has 100-fold selectively towards PLK1 and PLK3 (2.2 nM and 9.1 nM) compared to other kinases. The increased sensitivity required 500 nM treatment to affect cell growth in TRAMR cells and is therefore likely to be due to inhibition of off
target kinases. This also applies to the antagonistic affects with low-level trametinib treatment and high GW843682X dosage synergism (Figure 4.3 B).

### HCT116

<table>
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### TRAMR

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**Figure 4.2. Drug combination dose matrix.** Layout of a dose matrix experiment with Aurora kinase inhibitor ZM447439. HCT116 cells or their resistant sister cells were treated with a log-dose range of two compounds independently of each other (far left column and top row). Other wells were treated with every combination of the two compounds dose range. Cells were then incubated for 48 hours and cell viability analyzed using PrestoBlue™. Comparison of concentration combinations were then compared to the singular compounds to determine synergistic and antagonistic effects. Further comparison between the sister cells identified combinations or targets pertinent to MEK inhibition resistance.
ZM447439 is a potent inhibitor of Aurora A and B kinases (Ditchfield et al., 2003) and had an altered combinatorial effect with trametinib treatment (Figure 4.3 C, D). In trametinib sensitive HCT116 cells Aurora inhibition had little effect on cell viability as a single agent (Figure 4.3 C), while high dose ZM447439 (5-10 μM) is synergistic at low trametinib levels; this is lost at higher doses. This suggests that trametinib has a dominant effect in this combination. In trametinib resistant HCT116 cells (TRAMR) Aurora inhibition had a moderate effect as a single agent. The combination with trametinib demonstrates combinatorial synergism, limiting cell viability at higher concentrations to a similar level to sensitive HCT116 cells (Figure 4.3 C, D). Together this indicates a minor role for Aurora kinase in acquired resistance to trametinib.

UNC642 is a highly selective inhibitor of G9a and GLP methyltransferases (IC50 <2.5 nM). It has >2,000 times selectivity toward these H3K9 mono- and di- methyltransferases compared with H3K27me3 HMT Ezh2 and H3K9me3 HMT Suv39h enzymes (F. Liu et al., 2013). High dose treatment of G9a/GLP inhibitor UNC642 resulted in low levels of cell viability in both HCT116 and TRAMR cells (Figure 4.3 E, F). At low doses UNC642 promotes cell growth as a single agent in HCT116 cells (Figure 4.3 E). However, there is no significant synergism or antagonism with trametinib treatment. This suggests that H3K9me2 does not have a significant role in ERK driven cell proliferation. In trametinib resistance cells, UNC642 combination is highly antagonistic (Figure 4.3 F) and suggests that H3K9me2 has an altered function in cells that have undergone the EMT-like transition to acquire trametinib resistance. Proliferation within these conditions is enhanced over controls, suggesting that in TRAMR cells this function of H3K9me2 limits cell growth when proliferative MAPK signalling is reduced.
Figure 4.3. Trametinib 3D HSA dose matrix plots for kinase inhibitors. Fraction affected 3D dose combination plots overlaid with the HSA model for synergism with MEK inhibitor trametinib. HCT116 or TRAMR cells were treated with a log-dose matrix between trametinib and another compound for 48 hours followed by PrestoBlue™ cell viability assay. (A-B) Combination plots of for PLK inhibitor GW843682X. (C-D) Combination plots for Aurora kinase inhibitor ZM47439. (E-F) Combination plots of G9a, GLP inhibitor UNC642.
H3K9me2 has been implicated in the formation of facultative heterochromatin during differentiation at genic regions in addition to its deposition pericentromerically (Wen et al., 2009). Recent work has indicated that H3K9me2 functions to allow transcriptionally permissive pericentric heterochromatin, maintained by argonaute mediated transcriptional silencing (Jih et al., 2017). Maintenance of pericentric heterochromatin in the absence of RNAi mechanisms requires the transition to H3K9me3, demonstrating an independent role for H3K9me2 in heterochromatin formation. The antagonistic effect of G9a/GLP inhibition in TRAMR cells, therefore suggests that H3K9me2 has altered function in ERK signalling response after acquired resistance, that may indicate altered heterochromatin regulation and/or altered heterochromatic transcription.

Polycomb mediated silencing via the formation of facultative heterochromatin, is known to also function in differentiation mediated gene regulation as an alternative to H3K9me mediated heterochromatin (Gilbert et al., 2003). To determine if polycomb functions in trametinib acquired resistance, HCT116 and TRAMR cells were treated with selective inhibitors of Ezh2 (PRC2 subunit) and L3MBTL (PRC1 subunit), GSK343 and UNC 699 respectively (Figure 4.4). For both inhibitors in sensitive HCT116 cells, inhibition had no effect on proliferation until the highest dose of 10 μM; additionally there was no combinatorial effect with trametinib in HCT116 cells (Figure 4.4 A, C). However, the trametinib resistant cells, TRAMR, showed increased sensitivity to both inhibitors when used as single agents. This effect is accentuated in UNC 699 treated TRAMR cells suggesting that PRC1 and subsequent PRC2 recruitment may be altered.
In combination with trametinib both inhibitors, GSK343 and UNC 699, have antagonistic effects at low concentrations (Figure 4.4 B, D). This suggests that MAPK signalling interacts with polycomb regulation after developing MAPKi resistance. Canonical PRC1 complexes have a tight association to PRC2 and H3K27me3, and the common subunit Cbx7 is known to be phosphorylated by MAPK signalling (Kassis and Brown, 2012; Wu et al., 2013). This would suggest a synergistic relationship with MAPK inhibition. However, atypical PRC1 complexes with Cbx6 and L3MBTL2 subunits are known to be essential for ES cell differentiation functioning independently of PRC2 and repressing MAPK expression (Qin et al., 2012; Santanach et al., 2017). Together these data suggest that inhibition of PRC1 subunits would need to be highly specific in order to target a single PRC1 function.
Figure 4.4. Trametinib 3D HSA dose matrix plots for polycomb inhibitors. Fraction affected 3D dose combination plot overlaid with the HSA model for synergism with MEK inhibitor trametinib and polycomb subunit inhibitors. HCT116 or TRAMR cells were treated with a log-dose matrix of trametinib and another compound for 48 hours followed by PrestoBlue™ cell viability assay. (A-B) Combination plots for EZH2 inhibitor GSK343. (C-D) Combination plots for L3MBTL inhibitor UNC699.
PRC2 is also known to have both tumour suppressor and oncogenic functions depending on the tumour type. Many tumours overexpress PRC2 proteins including breast and colon (Bracken et al., 2003), but in contrast recurrent somatic loss of function mutations of PRC2 components are enriched in diffuse large B cell lymphoma and acute lymphoblastic leukaemia (Ernst et al., 2010). Subsequent studies have demonstrated that PRC2 loss in solid tumours has cooperative effects with MAPK signalling in driving tumour progression (De Raedt et al., 2014; Zhang et al., 2014). Taken together this suggests that PRC2 has tumour suppressor functions specifically acquired when developing trametinib resistance.

Intriguingly, Thomas De Raedt's (2014) work also suggests that loss of PRC2-MAPK tumour suppressor functions results is linked to BET protein function. To investigate this HCT116 and TRAMR cells were treated with multiple BET inhibitors to determine the function of BET proteins in trametinib resistance development (Figure 4.5). JQ1, I-BET151, and I-BET762 are selective inhibitors of Brd2, Brd3, Brd4 and Brdt (Filippakopoulos et al., 2010; Nicodeme et al., 2010; Mirguet et al., 2013). While selectivity differs between compounds, in HCT116 cells, BET inhibition has moderate affect as a single compound (Figure 4.5 A, C, E). Low dose of IBET762 and IBET151 was antagonistic to trametinib, and, although this only occurred at one dose, it was reproducible (n=3). This antagonistic effect aside, there was no combinatorial effect with trametinib in HCT116 cells. Resistant TRAMR cells were more sensitive to all three BET inhibitors as single agents (Figure 4.5 B, D, F) while JQ1 was highly antagonistic in combination with trametinib. IBET151 and IBET762 by contrast showed moderate antagonism with trametinib at higher dosages. Unlike JQ1, which is a pan inhibitor across the
BET proteins, IBET151 and IBET762 have differing selectivity across the class. IBET151 has decreased selectivity towards Brd2 and further decreased selectivity towards Brd3 \textit{in vitro}. IBET762 is most selective towards Brd2 followed closely by Brd4, and decreased binding to Brd3 \textit{in vitro} (Dawson \textit{et al.}, 2011). This suggests that the antagonistic properties come either from inhibiting all three BET proteins or is specifically linked to Brd2 and Brd3 rather than canonical Brd4 inhibition.
Figure 4.5. Trametinib 3D HSA dose matrix plots with BET inhibition. Fraction affected 3D dose combination plot overlaid with the HSA model for synergism with MEK inhibitor trametinib and BET inhibitors. HCT116 or TRAMR cells were treated with a log-dose matrix with trametinib and a BET inhibitor for 48 hours followed by PrestoBlue™ cell viability assay. (A-B) combination plots for JQ1\textsuperscript{157}. (C-D) combination plots for I-BET151. (E-F) Combination plots for I-BET762.
BET proteins bind to acetylated chromatin via tandem bromodomain hydrophilic pockets (Dhalluin et al., 1999). JQ1, IBET151 and IBET762 act as competitive inhibitors to acetylated lysines releasing BET proteins from chromatin without affecting protein structure. To determine whether acetylation changes were affecting BETi sensitivity, HCT116 and TRAMR cells were treated with a dose matrix of HDAC inhibitor trichostatin A and JQ1 (Figure 4.6). In HCT116 cells TSA and JQ1 are highly synergistic, indicative of inhibitors targeting the same pathway (Figure 4.6 A). Trametinib resistant cells sensitivity to the TSA single agent treatment was similar to that of HCT116 cells. However, JQ1-Trametinib combinations had a weak antagonistic effect, suggesting altered BET protein function after acquiring trametinib resistance (Figure 4.6 B). As TSA is a pan HDAC inhibitor this could be due to altered acetylation of non-chromatin substrates or acetylation independent recruitment of BET proteins. Recent work has demonstrated that structural chromatin protein CTCF can recruit Brd2 to determine transcriptional and architectural boundaries (Hsu et al., 2017). Additionally BET proteins Brd2 and Brd4 c-terminal domain interaction with viral DNA is known to cause recruitment to heterochromatin (You et al., 2004; Viejo-borbolla et al., 2005).
Figure 4.6. Trichostatin 3D HSA dose matrix with BET inhibition. Fraction affected 3D dose combination plot overlaid with the HSA model for synergism with HDAC inhibitor trichostatin A (TSA). HCT116 or TRAMR cells were treated with a log-dose matrix with TSA and JQ1 +/- for 72 hours followed by PrestoBlue™ cell viability assay. (A) combination plot of for HCT116 cells. (B) combination plot of for TRAMR cells.

To investigate which BET proteins or bromodomains were implicated in trametinib-acquired resistance, further BET inhibitors were compared (Figure 4.7). OTX015 is another pan BET inhibitor similar to JQ1 with little selectivity between Brd2, Brd3 and Brd4 (Coudé et al., 2015). RVX-208 by contrast has 10-100 fold selectivity to BET proteins second bromodomain. Additionally RVX-208 has decreased selectivity towards Brd4 when compared to Brd3 and Brd2 (Picaud et al., 2013). All BET inhibitors apart from RVX-208 demonstrated increased sensitivity in TRAMR cells, indicating that both bromodomains are essential to this acquired function. The order of sensitivity across the BET inhibitors also correlates with selectivity towards Brd3 and Brd2 in contrast to Brd4 suggesting that one or both of these proteins may be involved in the acquired resistance to MAPK inhibition.
Figure 4.7. BET inhibition and cell viability. Bar charts showing cell viability using a PrestoBlue™ assay. HCT116 and TRAMR cells were treated with a half-log dose curve of BET inhibitors for 48 hours, independently. Viability was determined by Presto Blue reagent incubation and 540/590 nm measured on a Victor3 multi-label plate reader.
4.4 The role of BET proteins in acquired resistance to ERK inhibition

In order to further investigate the function of BET proteins, both HCT116 cells and TRAMR were treated with increasing doses of JQ1 up to 1 µM over three months to develop resistance also known to occur in patients (Kurimchak et al., 2016). HCT116 and TRAMR cells resistant to JQ1 treatment (HJQ1R and TJQ1R) were treated with dose curves of trametinib +/- 1 µM JQ1, and JQ1 as a single agent along with their parental cell lines (Figure 4.8). With trametinib treatment HCT116 cells were the most sensitive. Intriguingly, both JQ1 resistant cell lines had similar tolerance of trametinib, beyond that of TRAMR cells (Figure 4.8 A). When treated with a log dose range of JQ1, both JQ1 resistant cell lines demonstrated negligible reduction. Reaffirming the drug combination matrix results TRAMR cells were more sensitive to JQ1 treatment than HCT116 cells (Figure 4.8 B).
1 µM JQ1 combined with a log dose range of trametinib was however synergistic in both JQ1 and trametinib resistant cells (Figure 4.8 C) which suggests that BET inhibition is able to overcome MEK inhibition, reverting resistant cells to a sensitive state. Additionally, the synergism of JQ1 and trametinib in JQ1 resistant cells suggest that these act as a switch between molecular states, and not just as an upregulated multidrug resistance pathway. Together these results suggest that BET/MEK inhibition would be a viable drug combination, as they prevent acquired resistance to each other. This hypothesis has been further established by recent studies demonstrating that a Tip60/Brd4/Twist complex is required for nuclear reorganisation in an endothelial mesenchymal like transition (Shi, Cao and Zhou, 2015).

The antagonistic combination shown previously in figure 4.5 B was not observed here at these comparatively higher dosages. Hyperphosphorylation at multiple protein residues is known to reduce the affinity of trametinib to MEK1, decreasing its efficacy, resulting in the stabilisation of phosphorylated c-myc, preventing its ubiquitination (Gilmartin et al., 2011; Tsai et al., 2012). Subsequent studies have demonstrated that c-myc, known to be regulated by BET proteins, contributes ERBB2/3 suppression, thus acting as a negative feedback loop (C. Sun et al., 2014). As such, BET inhibition and downstream reduction of c-myc will partially compensate for trametinib MEK1 inhibition. Further studies demonstrated that more extensive RTK inhibition was, however, able to overcome this myc driven feedback loop, demonstrated by higher doses used in figure 4.8 C (Lito et al., 2012).
Figure 4.8. Development of JQ1 resistance in combination with trametinib. Comparison of HCT116 cells and their sister cells with trametinib or JQ1 acqired resistance cells. Cell viability was assayed by PrestoBlue™ incubation and measured at 540/590 nm. (A) Cells treated with a log-dose range of trametinib for 24 hours. (B) Cells treated with a log-dose range of JQ1 for 24 hours. (C) Cells were treated with a singular dose of 1 µM JQ1 and a log-dose range of trametinib for 24 hours.
BET proteins have multiple functions modulating cell cycle progression. Brd4 stays bound to chromatin through mitosis, and is known to interact with condensin II and insulate chromatin from the DNA damage response. Loss of this function induces relaxed chromatin compaction and rapid cell-cycle checkpoint recovery (Floyd et al., 2013). Brd2 was first identified as a regulator of cyclin A transcription and subsequent cell cycle progression (Sinha, Faller and Denis, 2005). To determine whether cell cycle alterations occur when HCT116 cells develop resistance to trametinib and JQ1 that could alter BET inhibition efficacy, propidium iodide cell cycle flow cytometry analysis was performed (Figure 4.9). HCT116, TRAMR, and HJQ1R cells were tested with 1 µM JQ1 for 48 hours. Untreated TRAMR cells had a similar cell cycle profile to untreated HCT116 cells, suggesting that the development of resistance to trametinib had no effect on cell cycle progression. Untreated HJQ1R cells however had elevated levels of cells in S phase and G2 indicating that the development of JQ1 resistance alters cell cycle progression. In all cell lines however JQ1 treatment resulted in an increase of cells in G1 (Figure 4.9 G) and suggests that development of resistance to JQ1 is an inherent property of “resistant” cells and not just an altered response to JQ1 treatment.
Figure 4.9. BET inhibition and the cell cycle. Frequency distributions for propidium iodide (PI) stained cells analyzed by flow cytometry. Treated cells were ethanol fixed and stained with 1 µg/ml of PI for 2 hours at room temperature. PI fluorescence was detected at 695-740 nm by flow cytometry (A-B) HCT116 cells treated with DMSO or 1 µM JQ1+/− for 48 hours (C-D) TRAMR cells treated with DMSO or 1 µM JQ1+/− for 48 hours (E-F) HJQ1R cells treated with DMSO or 1 µM JQ1+/− for 48 hours. (G) Cell cycle stage ratios from A-F.
To determine if there was a change to the spatial distribution of Brd4, Brd4 was analysed by immunofluorescence after 1 µM JQ1 treatment for 24 hours (Figure 4.10). In untreated HCT116 and TRAMR cells Brd4 had a diffuse staining pattern but HJQ1R cells by contrast had a diffuse staining pattern in addition to some punctate spots. The appearance of punctate Brd4 also occurred in each cell line after JQ1 treatment. To determine if Brd4 was redistributed from a central to more peripheral position, DAPI images were used in conjunction with edge segmentation to determine nuclear boundaries for each nucleus. These were then applied to the linked Brd4 image and the average radial pixel intensity was calculated and binned into deciles for each cell. At least 20 cells were analysed for each treatment and cell line and plotted (Figure 4.10 B-G). Both HCT116 and TRAMR untreated cells had Brd4 radial distributions weighted to more central positions, in line with canonical functions of Brd4 within euchromatin, and JQ1 treatment in HCT116 cells did not significantly alter Brd4’s distribution (Figure 4.10 B-C). TRAMR cells by contrast saw a significant shift in Brd4 signal towards the periphery, which could be indicative of Brd4 being recruited to heterochromatic regions (Figure 4.10 D-E). HJQ1R cells differed from HCT116 and TRAMR cells as untreated Brd4 was evenly distributed from the centre to the periphery. JQ1 treatment of HJQ1R cells also did not significantly alter this distribution despite the signal being more variable (Figure 4.10 F-G) which might suggest that a Brd4 distribution associated with the nuclear periphery is not dependent on bromodomain function.
Figure 4.10. Brd4 distribution in nucleus after BET inhibition. HCT116, TRAMR and HJQ1R cells were treated with DMSO or 1 µM JQ1 +/- for 24 hours. Cells were washed before fixation to remove unbound proteins, and stained with DAPI and Brd4 antibodies. (A) DAPI and Brd4 images of cell nuclei. (B-G) Radial distribution of Brd4 pixel signal intensity binned into deciles from more central positioning to the nuclear periphery. (B-C) HCT116 cells treated with DMSO or JQ1 +/- (D-E) TRAMR cells treated with DMSO or JQ1 +/- (F-G) HJQ1R cells treated with DMSO or JQ1 +/-.
To determine if the increased sensitivity to JQ1 in TRAMR cells was directly due to BET protein transcription, RT-qPCR normalised to MRIP was used to analyse RNA levels (Figure 4.11). In HCT116 cells, JQ1 1 µM treatment for 6 hours resulted in no significant changes in Brd3 and Brd4 transcription, with a minor decrease in Brd2. C-myc, the canonical downstream effector of Brd2 and Brd4, also showed no significant increase in transcription after JQ1 treatment. Untreated TRAMR cells by comparison had a 4-fold increase in c-myc transcription. This could implicate c-myc as a driver of resistance within TRAMR cells. Importantly, this increase in c-myc transcripts is lost upon JQ1 treatment, directly implicating BET proteins in regulating MEK inhibition resistance. Untreated TRAMR cells also had a 2-fold increase in Brd2 transcription. JQ1 treatment did not affect Brd3 and Brd4 transcription but Brd2 decreased 4-fold. This suggests that the only immediate change in transcription post JQ1 treatment is a reduction in Brd2.

![Graph showing transcriptional response to BET inhibition](image)

**Figure 4.11. Transcriptional response to BET inhibition.** HCT116 and TRAMR cells were treated with DMSO or 1 µM JQ1 for 24 hours. Transcription of c-Myc, Brd4, Brd3, and Brd2 was quantified by qPCR. Amplification was normalized to the housekeeping gene MRIP.
RT-qPCR was also used to determine if these transcriptional changes were sustained out to 24 hours and determine the effect on HJQ1R cells (Figure 4.12). HCT116 and TRAMR BET protein transcription broadly mirrored the changes at 6 hours, except a two-fold reduction in Brd2 after JQ1 treatment in HCT116 cells. Untreated HJQ1R cells had no significant changes in all BET proteins when compared to HCT116 cells. JQ1 treatment however resulted in decreased transcription (30-70%) across the BET proteins. Intriguingly, 7sk RNA levels were also elevated in TRAMR cells. 7sk functions in a complex to sequester P-TEFb, regulating its binding to Brd4 and its function in transcriptional elongation (Prasanth et al., 2010). Together this suggests that BET proteins have altered post-transcriptional regulation in TRAMR cells.

The radial distribution of Brd4 in TRAMR cells after JQ1 treatment and HJQ1R distribution suggests a role at the nuclear periphery (Figure 4.10). HP1 proteins play an important role in the maintenance and propagation of heterochromatin (Nakayama, Klar and Grewal, 2000; Hall et al., 2002). Therefore RT-qPCR was used to determine if BET proteins regulate heterochromatin via HP1 proteins (Figure 4.12). Upon JQ1 treatment HCT116 cells showed no significant change to HP1α transcripts, while HP1β and HP1γ are elevated by 48% and 68%, respectively. Untreated TRAMR cells however showed no major change in any HP1 isoform transcripts after JQ1 treatment or to HCT116 cells. Untreated HJQ1R by contrast had decreased levels of HP1α accentuated by JQ1 treatment. HP1β was also reduced after JQ1 treatment in HJQ1R cells suggesting that acquiring resistance to JQ1 alters HP1 transcription.
Figure 4.12. HP1 α/β/γ protein expression in response to BET inhibition. HCT116, TRAMR, and HJQ1R cells were treated with 1 μM inactive JQ1 or active JQ1 for 24 hours. Transcription of 7SK, BET proteins and HP1 proteins were quantified by qPCR. Amplification was normalized to the house-keeping gene MRIP.
Western blotting was used to assess whether JQ1 treatment and acquired resistance affected protein levels (Figure 4.13) and densitometry was performed to quantify protein levels in HCT116, TRAMR and HJQ1R cells. Brd4 total protein levels were only elevated in HJQ1R cells upon JQ1 treatment. This indicates that HJQ1R cells may overcome JQ1 treatment by preventing Brd4 degradation, as transcription is not suppressed (Figure 4.12). Brd3 nuclear protein levels were stable across all three cell lines and treatment conditions, suggesting that it is not involved in acquired resistance to BET or MEK inhibition but Brd2 nuclear protein levels by comparison were altered in the resistant cell lines. Although HCT116 cells showed no major changes in Brd2 levels after JQ1 treatment, TRAMR cells presented with a significant reduction. Untreated HJQ1R cells also had significantly lower Brd2 levels when compared to TRAMR and HCT116 cells. Upon JQ1 treatment however Brd2 levels are restored to levels similar to HCT116 and TRAMR cells. Taken together this implicates Brd2 in the phenotypic switch between JQ1 and trametinib resistance implicated in figure 4.8.
Figure 4.13. Nuclear protein levels after BET inhibition. (A) Westerns of HCT116, TRAMR, and HJQ1R nuclear lysates after 48 hour treatment with DMSO or JQ1+/- 1uM. (B) Bands were quantified by 2D densitometry and normalised to total histone H3. Relative protein levels determined by comparison to HCT116 DMSO controls.
HP1 nuclear protein levels were also analysed by western blotting (Figure 4.13). JQ1 treatment in HCT116 cells had no effect on any HP1 isoform nuclear concentration. Untreated TRAMR cells presented elevated levels of HP1α and HP1γ when compared to HCT116 cells and this effect was partially negated by JQ1 treatment. Untreated HJQ1R cells also had increased HP1α nuclear levels, but no significant changes to HP1β or HP1γ levels. In contrast, JQ1 treatment in TRAMR cells resulted in a further increase in HP1α levels. This suggests that HP1α may function in acquiring resistance to JQ1 and trametinib in addition to Brd2. Intriguingly, H3K9me3 levels appear to be severely depleted in HJQ1R cells. As H3K9me3 and HP1α are hallmarks of pericentric heterochromatin, this also suggests an altered chromatin state at the nuclear periphery in response to developing resistance.

![Figure 4.14](image)

**Figure 4.14. Transcriptional response to trametinib resistance.** Global transcription levels determined by the incorporation of 5 µl per ml of [5-3H]-uridine within 30 minutes. HCT116, TRAMR, and HJQ1R were treated with DMSO or 1 µM JQ1 for 24 hours prior to [5-3H]-uridine exposure (n = 3 technical replicates). RNA was purified and incorporation determined via scintillation counting (n = 2).
HP1 proteins have been associated with RNA at pericentric heterochromatin, and are known to have a non-specific RNA binding site. To determine if global transcription levels were affected by JQ1 or resistance the incorporation of tritiated thymidine was assayed. 5 µl [³H]-Thymidine was added per ml of media for each sample for 30 min to allow incorporation into RNA polymerase transcripts. Purified RNA was added to scintillant and measured on a scintillation counter (Figure 4.14). While biological replicates showed some variation, there was no consistent effect of JQ1 on global transcription in any cell type, for this short period of time. Untreated HJQ1R cells showed a minor increase (30%) in global transcription levels compared to HCT116 cells, however given the variation in untreated HCT116, this was not likely to be significant. Untreated TRAMR cells in contrast have a 2-fold increase in global transcription levels. This suggests that in addition to elevated Brd2 and 7sk levels (Figure 4.12), there is a significant but subtle global change in transcriptional regulation after acquiring trametinib resistance.
4.5 Discussion

Epithelial to mesenchymal transitions (EMT) are driven by specific transcription factors SNAIL and TWIST, and typified by the loss of E-cadherin (Cano et al., 2000; Yang et al., 2004). Rapid development of acquired resistance to MAPK inhibitors is associated with an EMT-like transition across tumour types (Byeon et al., 2017; Kitai and Ebi, 2017) and EMT tumour progression is known to result in genome wide chromatin reorganisation. This chromatin reprogramming encompasses distinct alterations of H3K9 methylation and HP1 binding to heterochromatin (McDonald et al., 2011; Millanes-Romero et al., 2013). Therefore, the inhibitors identified as modulating synthetic heterochromatic domains (Table 3.1), were investigated within a MAPK inhibitor resistant model system.

The initial studies characterising the altered phenotype to prolonged MEK inhibition (Figure 4.1) demonstrated that this in vitro cell line model mirrored the patient phenotype for overexpression/hyperphosphorylation of the Raf/Ras/MEK/ERK pathway (Montagut et al., 2008; Long et al., 2014). Trametinib resistance resulted in more invasive cells and basal levels of ERK phosphorylation were upregulated, whilst the adaptive signalling response was tempered in trametinib resistant cells (Table 4.1).
To determine if drugs could modulate pathways associated with an EMT-like transition to a drug resistant state, they were screened in a dose-matrix combination with MEK inhibitor trametinib (Figure 4.2). Two specific kinase inhibitors of PLK and Aurora B (Figure 4.3) were identified as having an altered phenotypic effect in MEKi resistant cells. PLK, a known regulator of chromosome segregation correlates with tumour grade and invasiveness across Ras driven tumours (X. G. Zhang et al., 2012; W. Sun et al., 2014) and the elevated sensitivity towards PLK inhibition suggests increased dependence on PLK in MEKi resistant cells. PLK is also a downstream effector of Akt driven EMT, while hyperphosphorylation of Ras has been linked to Akt crossover activation (Carracedo and Pandolfi, 2008; Cai et al., 2016; Ferrarotto et al., 2017). The antagonism between GW843682X and trametinib however suggests that trametinib promotion of EMT and acquired MEKi resistance is not dependent on PLK phosphorylation.

Aurora kinases also plays a key role in chromosome segregation during mitosis, with Aurora A being implicated in activating EMT (D’Assoro et al., 2014). Increased sensitivity to Aurora A/B inhibition by ZM447439 as a single agent in TRAMR cells is therefore expected. Additionally the weak synergism with trametinib treatment is likely due to the direct activation of Aurora A by MAPK signalling previously reported, further demonstrating the dependence of EMT and heterochromatin regulation in acquired drug resistance (Wan et al., 2008).
Three epigenetic target classes (EZH2, L3MBTL, and BET) from the inhibitor panel (Table 3.1) also had altered sensitivity in TRAMR cells. UNC642, a potent inhibitor of G9a/GLP methyltransferases was more sensitive as a single agent in resistant cells, but had significant antagonism with MEK inhibition (Figure 4.3). G9a/GLP are responsible for H3K9 mono- and di-methylation. These histone modifications are thought to regulate the formation of facultative heterochromatin and gene silencing via HP1 recruitment (Ogawa et al., 2002; Lyons et al., 2014). G9a has previously been associated with EMT-like phenotype by interacting with SNAIL in MAPK driven tumours (Dong et al., 2012). This suggests that G9a/GLP inhibition would be synergistic with trametinib. However, G9a has been identified as a direct regulator of Sprouty4 which in turn negatively regulates MAPK signalling and the mesenchymal phenotype (Taniguchi et al., 2009; Hua et al., 2014). This regulation is likely to contribute to large antagonistic effect with G9a/GLP-MEK combinations.

The EZH2 inhibitor, GSK343, demonstrated a weak increase in response to treatment in TRAMR at high concentrations only (Figure 4.4 B). Equally, in combination with trametinib it showed a weak antagonistic effect. UNC699, an inhibitor of the PRC1 subunit, L3MBTL, showed a large increase in response to treatment as a single agent at low dosage, by contrast. However, UNC 699 was also antagonistic in combination with trametinib (Figure 4.4 D). As discussed previously, canonical and atypical PRC1 complexes have been linked with driving EMT and repressing MAPK signalling, demonstrating its dual role and subsequently antagonism with MEK inhibition (Kassis and Brown, 2012; Santanach et al., 2017).
BET protein inhibitors were also identified as having increased sensitivity in TRAMR cells (Figure 4.5) and were antagonistic in combination with trametinib. Recent studies have demonstrated that RTK-driven cancers’ resistance mechanism converge on MYC activation supporting this result (Ma et al., 2017; Singleton et al., 2017; Zawistowski et al., 2017). These studies also present evidence that MEK inhibition is enhanced with the addition of JQ1, however they did not comprehensively analyse the synergism of the combinations. While BET-MEK combination therapy may not be synergistic, the additive effects presented in these studies and ability to resensitise MEKi resistance was demonstrated at higher trametinib concentrations (Figure 4.8). Singleton, et al (2017) and Zawistowski, et al (2017) studies were also performed on in vivo models over prolonged treatment which would accentuate any cytostatic effects of the combination. This was not be identified with the combination matrixes analysed in figure 4.5 or figure 4.8 which were performed over 24-48 hours treatment but these studies do, however, validate findings that BET inhibition has altered effects in cells that have adaptive resistance to MAPK inhibition.

Variations in sensitivity towards different BET inhibitors gave an indication of which BET proteins had the dominant effect on acquired resistance (Figure 4.5 and 4.7). The results shown here suggest that selectivity towards Brd2 influenced cell survival. However Brd4 nuclear distribution was significantly altered after JQ1 treatment in TRAMR cells, with increased association with distinct loci at the periphery (Figure 4.10). No clear change in Brd2 pattern was observed (data not shown), suggesting that the change in Brd4 distribution occurs with general loss of Brd2. This interplay between Brd2 and Brd4 supports the current studies indicating a partial overlap in function (Cheung et al., 2017). Recent studies have also indicated that isoform
switching between Brd2 and Brd4 drives lineage specific differentiation (Fernandez-Alonso et al., 2017). Cell cycle analysis showed no difference between HCT116 and TRAMR cells suggesting that the atypical Brd4 distribution was not due to mitotic defects (Figure 4.9). The potential role of Brd2 was further linked to this response, attributable to its elevated transcription in TRAMR cells. Supporting the recent studies indicating MYC-driven resistance to MAPK inhibition, c-myc was also elevated in TRAMR cells; elevated levels of c-myc and Brd2 are both lost upon JQ1 treatment. Brd3 and Brd4 levels remained unchanged further implicating Brd2 as the driver of JQ1 sensitivity. Consistently, loss of Brd2 transcripts also translates to protein levels (Figure 4.13).

Intriguingly, HP1 isoform levels were significantly altered by acquiring trametinib resistance and JQ1 treatment. Untreated TRAMR cells had significantly increased abundance of nuclear HP1α and HP1γ (Figure 4.13) but JQ1 treatment caused significant reductions in all HP1 isoforms. This suggests that BET proteins play a significant role in regulating HP1 transcription and consistently in cells resistant to JQ1 treatment these changes to BET and HP1 proteins were broadly reversed, providing further
evidence of BET protein regulation in switching of phenotypic states.

Figure 4.15. BET proteins distribution at HP1 promoters. Brd4 ChIP-seq tracts from Asangani (2014), W. Liu (2013), and Knoechel (2014) on epithelial cell lines HEK292 and HeLa compared to mesenchymal drug resistant prostate cell line VCaP and T-ALL cell line DND41-persistors. Black bar highlights the promoter region of HP1γ.
Comparison of publically available Brd4 ChIP-seq data sets further supports this position. Epithelial cell lines HEK293 and HeLa (W. Liu et al., 2013) lack Brd4 binding at HP1 promoters while mesenchymal and drug resistant DND-41 and vCaP cells (Asangani et al., 2014; Knoechel et al., 2014) have distinct Brd4 peaks over HP1 promoters (Figure 4.15). JQ1 treatment of the castration resistant VCAP cells resulted in a significant decrease in Brd2 occupancy over HP1 promoters when compared to Brd3 and Brd4, further suggesting a Brd2 specific role in acquired MAPK resistance (Figure 4.16).

Figure 4.16. BET chromatin binding at HP1 promoters with JQ1 treatment. ChIP-seq tracts from Asangani (2014), depicting Castration resistant mesenchymal prostate cancer cells VCaP. VCaP cells were treated with DMSO or 0.5 μM JQ1+/- and immune-precipitated for Brd2, Brd3, and Brd4. HP1β gene CBX1 shows reduced BET protein occupancy after JQ1+/- treatment, representative of the HP1 family. Black bar highlighting HP1β promoter.
Together the results shown here further support the concept that relates EMT-like phenotypes with acquired MAPK inhibitor resistance, and the identification of BET proteins as a potential therapeutic agent to resistant cell types. Additionally, this work suggests that BET proteins are associated with heterochromatin regulation in acquired MAPKi resistance. There is evidence suggesting that Brd2 drives BET proteins function at heterochromatin. This could be indirect via regulating HP1 transcription or direct by TIP60 mediated recruitment to pericentric repeats (Grézy et al., 2016). Although the regulation of pericentric heterochromatin, is not well understood, TIP60 is known to acetylate H4K12 at satellite repeats, recruiting Brd2 to maintain chromatin compaction and suppress transcription, while loss of Brd2 association via BET inhibition leads to aberrant satellite expression that could explain the Brd4 punctate recruitment to the nuclear periphery (Figure 4.10). Investigations of heterochromatin compaction and its impact on cellular phenotype will provide an exciting avenue of future research.
Chapter 5: The role of H3K9me3 in heterochromatin regulation

5.1 Introduction

A well-defined model of constitutive heterochromatin is mouse embryonic stem cells (mESCs). Mouse centric and pericentric heterochromatin is easily visualised by DNA staining, as it co-locates to form chromocenters unlike most primate cells. Pericentromeric regions are comprised of A-T rich repetitive elements such as minor and major satellites in mouse cells; major satellites form large arrays of a 234 bp core monomer (Vissel and Choo, 1989), whilst minor satellite forms a 120 bp repeat. Due to the role of centromeric chromatin in correct kinetochore assembly, chromatin abnormalities in constitutive heterochromatin can lead to mitotic defects (Ekwall et al., 1997). In Drosophila and mammalian cells constitutive heterochromatin also spreads to adjacent euchromatic regions mediating transcriptional suppression (Muller, H, 1930) and position effect variegation. The constitutive heterochromatin at these suppressive regions of variegation are characterised by DNA methylation, general hypoacetylation, H3K9 methylation and association of HP1 proteins (James and Elgin, 1986; Jeppesen et al., 1992; Jeanpierre et al., 1993; Peters et al., 2001).
The interplay between HP1 proteins, H3K9me3, and Suv39h1/2 methyltransferases has been extensively studied at constitutive heterochromatic regions. HP1 isoforms are highly conserved across species from Swi6 in *S. pombe* to HP1α/β/γ in mammalian cells (Singh *et al.*, 1991). HP1 proteins are regulators of gene expression by position-effect variegation (Eissenberg *et al.*, 1990). Although they do present some variation in their distribution (Minc, Courvalin and Buendia, 2000; Nielsen *et al.*, 2001), the differential functions of the HP1 isoforms have yet to be fully elucidated. The HP1 protein encodes a chromo domain that facilitates its binding to H3K9me3 (Jacobs and Khorasanizadeh, 2002; Nielsen *et al.*, 2002) in a process that requires dimerisation of HP1 proteins via its chromoshadow domain (Cowieson *et al.*, 2000; Sadaie *et al.*, 2008). Subsequent studies on the *S. pombe* HP1 homologue Swi6 suggest that proximity of two H3K9me3 enables two HP1 proteins to tetramerise on a nucleosome which, in addition to the formation of homodimers, enables oligomerisation of HP1 across large H3K9me3 domains facilitating heterochromatin propagation (Canzio *et al.*, 2011).
The disordered hinge region of HP1 proteins is the least conserved and has been shown to interact with both RNA and DNA (Meehan, Kao and Pennings, 2003; Keller et al., 2012). As RNase treatment triggers the dissociation of HP1 from heterochromatin (Maison, Bailly, Peters, J.-P. Quivy, et al., 2002) it suggests an RNA component to HP1 recruitment or stabilisation. In *S.pombe* DICER mediated RNAi produces short transcripts from pericentric regions that stabilise the recruitment of HP1 proteins, while in humans lncRNA TERRA has been implicated in HP1 recruitment to telomeric constitutive heterochromatin, however a similar mechanism at pericentric heterochromatin has yet to be found (Arnoult, Van Beneden and Decottignies, 2012; Keller et al., 2013).

HP1 canonical recruitment to heterochromatin is driven by Suv39 histone methyltransferase through the deposition of H3K9me3. This was proposed when Suv39h double null knockout fibroblast cells showed a loss of H3K9me3 and HP1 from heterochromatin (Lachner et al., 2001). However, HP1 affinity for trimethylated H3K9 is particularly low suggesting that additional factors are directly involved in its recruitment (Jacobs and Khorasanizadeh, 2002). This is further supported by studies on the X-chromosome where inactivation leads to H3K9me3 deposition but not HP1 recruitment (Chadwick and Willard, 2003), but to complicate the picture Suv39h1 and Suv39h2 have also been implicated in directly recruiting HP1 proteins (Hall et al., 2002). Due to the cooperativity between H3K9me3 deposition, HP1 and Suv39h recruitment, recent studies have focused on identifying the causal seeding event on unmethylated H3K9 domains.
Suv39h methyltransferases contain an N-terminal chromodomain and a C-terminal SET domain (Horita et al., 2001; Min et al., 2002) which are highly conserved across species and protein classes. SET domains are catalytic sites for S-adenyl-methionine (SAM) mediated methylation (Trievel et al., 2002). whereas chromodomains were identified as a consensus motif between polycomb and HP1 proteins in *drosophila* (Paro and Hogness, 1991). Site directed mutagenesis experiments showed that these chromodomains functioned in gene repression (Messmer, Franke and Paro, 1992). This was confirmed by *in vitro* studies demonstrating their reactivity towards di- and tri-methylated lysines (Hughes et al., 2007). Suv39h chromodomains however are more specific towards trimethylated states (Schalch et al., 2009). This specificity however suggests that Suv39h chromodomain is not involved in its recruitment for *de novo* heterochromatin formation.

Given that RNase treatment leads to loss of the H3K9me3/HP1/Suv39h complex from heterochromatin, back to back studies in eLife suggested that recruitment of Suv39h enzymes can be mediated by RNA (Johnson et al., 2017; Shirai et al., 2017; Velazquez Camacho et al., 2017). However, these studies do not completely validate each others’ findings. They present conflicting data around the specificity of Suv39h binding to specific RNA transcripts and general RNA/DNA binding affinity. Despite the controversial claims of these studies, they do provide strong evidence for a direct interaction between Suv39h and nucleic acids, and that pericentric transcripts can associate with local chromatin and concentrations correlate with Suv39h association.
In addition to RNA and H3K9me3, HP1 SUMOylation has been implicated in regulating RNA associated de novo heterochromatin formation and maintenance (Shin et al., 2005). Maison et al. (2011) demonstrated that HP1 proteins are only associated with major satellite RNA transcripts when SUMOylated at the hinge region, and that this only occurs at low levels (>1%) in vivo. In Suv39h double null mouse cells, constitutively SUMOylated HP1α, HP1 was weakly associated with the pericentromere despite the lack of its binding partners H3K9me3 and Suv39h. However the exact method of de novo chromatin recruitment remains elusive, with recent evidence implicating Suv39h as recruiting Ubc9 to SUMOylate HP1 proteins (Maison et al., 2016). While SUMOylated HP1 proteins are chromatin associated at de novo heterochromatin sites, the SUMO protease SENP7 is required to maintain HP1 enrichment across established heterochromatin domains by reducing its mobility (Maison et al., 2012). SENP7 has two HP1 binding motifs that have been suggested to recruit SENP7 to sites of oligomeric HP1 in contrast to de novo HP1 recruitment, demonstrating how SUMOylation is removed from established heterochromatin (Romeo et al., 2015).

The interplay between H3K9me3, Suv39h, and HP1 in their binding of chromatin is complex; Suv39h double null cells also lose H4K20me3 and DNA methylation while presenting chromosome instability with increased tumour risk in mice (Peters et al., 2001). Knock out of DNA methylation enzymes by contrast does not result in the loss of Suv39h mediated H3K9me3 or chromatin compaction, implying that Suv39h is the master determinant of heterochromatin modifications (Martens et al., 2005; Gilbert, Thomson, Boyle, Allan, Ramsahoye and Wendy A Bickmore, 2007). Despite an absence of extensive epigenetic marks Suv39h−/− mice cells still form cytological chromocenters, questioning the perceived role of histone
modifications and their binding partners in being able to regulate the compaction state of the underlying chromatin. Therefore, before investigating the role of histone modifiers such as BET inhibitors on HP1 proteins and chromatin compaction, the regulators of chromatin compaction need to be further characterised.
5.2 Characterising the chromatin state of Suv39h double null mESC’s

To maintain mouse embryonic stem cells in an undifferentiated state, different growing conditions were first analysed (Figure 5.1). mESCs were grown on gelatin coated Corning orange capped flasks and yellow capped Sarstedt cell+flasks for sensitive adherent cell types. 10X magnification bright field images show that growth in yellow capped flasks resulted in more round distinct colonies, similar to the morphology of ES cells grown in 2i medium (information from Richard Meehan lab). This was regardless of Suv39h phenotype (Figure 5.1, A). Transcription of key pluripotency factors Nanog and Oct4 were then analysed by qPCR. Nanog and Oct4 levels were elevated in the orange capped Corning flasks indicative of increased stem cell characteristics (Chambers et al., 2003; Niwa, Miyazaki and Smith, 2016) (Figure 5.1, B). Therefore cells were grown on gelatinised corning flasks for cell line maintenance.
Figure 5.1. Mouse embryonic stem cell phenotype. (A) 10X bright field images of mouse ESC colonies from wild type [WT41] and Suv39h1/2 double knockout cells [DN57]. “Orange Caps” are Corning CellStar flasks manually coated in 0.1% gelatin for 5 minutes, “Yellow Caps” are Sarsted Cell+ pre-coated flasks. (B) RNA from mESC’s was harvested using an RNeasy kit and cDNA libraries prepared. QPCR for stem cell factors Nanog and Oct4, normalized to actin for cells grown on Corning orange capped flasks coated in gelatin and Sarstedt Cell+ yellow capped pre-coated flasks. Error bars = SEM of biological replicates (n=3).
To analyse the effect of Suv39h1 and Suv39h2 double knock out (DN57) on heterochromatic makers in ES cells, nuclei were isolated and analysed by western blotting compared to wild-type cells (WT41) (Figure 5.2). Double null cells (DN57) showed a 20% decrease in H4K20me3 and a significant increase in mono-methylation. This supports evidence linking Suv39h regulation of H4K20me3 at the pericentromere (Peters et al., 2001; Yang et al., 2008). H3K27me3 was also elevated in the knockout cells at the expense of H3K27me2. This suggests that polycomb activity is increased in the absence of Suv39h. Intriguingly, repressive H3K9 di- and tri-methylation were not completely lost. This suggests some redundancy with other methyltransferases at least on a global scale. HP1α and HP1β levels, were also reduced, possibly as a feedback from their disassociation from chromatin. Together these data suggest that Suv39 double knockout mESCs mimic phenotypes described previously (Peters et al., 2001).
Figure 5.2. Heterochromatic histone modifications response to Suv39h1/2 loss. (A) Nuclei were isolated and protein lysates prepared from WT41 and DN57 mouse ES cells. Samples were run on a 4-12% Bis-Tris Nupage gel and western blots performed for multiple heterochromatin components. (B) Densitometry quantification of the blots. Results show the relative levels of DN57 to WT41 samples.
As global levels of H3K9me3 and H4K20me3 were not completely lost in the double knockout cell lines, immune-fluorescence was used to determine their nuclear distribution (Figure 5.3). Both H3K9me3 and H4K20me3 signal in wild type cells were concentrated at chromocentres as visualised by DAPI staining and this was lost in the knock out cell lines.

To investigate changes in chromatin compaction at the level of the 30-nm chromatin fibre after loss of Suv39h, chromatin was analysed by sucrose gradient sedimentation and Southern blotting (Figure 5.4). This technique was used previously to measure compaction in a Dnmt3a/b null background (Gilbert et al., 2007). Nuclei were first isolated from the cells and digested with RNase A/T1 and micrococcal nuclease, in order to release soluble chromatin fibres from the nucleus under physiological salt conditions. In this approach RNase digestion is used to stop nuclear RNAs competing for binding of H1 to chromatin, which would otherwise affect the structure. However, this does have the disadvantage of degrading potential nuclear RNAs that might otherwise affect chromatin structure. In addition, micrococcal nuclease also attacks the 5’phosphodiester backbone of RNA so even in the absence of RNase the enzyme will digest potential chromatin associated RNAs and this might affect conclusions that have been drawn in studies implicating RNA in Suv39h recruitment (Cunningham, 1959; Velazquez Camacho et al., 2017). After soluble chromatin is released from the nucleus over-night it is fractionated on an isokinetic sucrose gradient in an ultra-centrifuge.
Figure 5.3. Distribution of histone modifications in SUV-/- cells. Wild type (WT41) and Suv39h1/2 knockout (DN57) mouse ES cells imaged for the methylation modifications attributed to Suv39h and Suv420h enzymes (H3K9me3, and H4K20me3). (A) images of DAPI and H3K9me3 immunofluorescence (B) Images of DAPI and H4K20me3 immunofluorescence. Scale bar is 20 µm.
The sedimentation rate of chromatin is determined by the fragment size (mass) and its structure. More disrupted chromatin fibres sediment at a slower rate than a more regular compact fibre of the same DNA length due to differences in friction. After sedimentation the gradient is fractionated by upwards displacement while measuring the DNA content via an inline UV spectrometer. Chromatin fibres of the same size (mass) but different compaction states will therefore be in different sucrose gradient fractions. Proteinase K treatment and phenol:chloroform extraction is then used to purify the DNA in each fraction before fractionating the DNA on a 0.7% Tris-phosphate gel at a low voltage to improve resolution (Figure 5.4, B).

In order to detect specific DNA regions the gel is Southern blotted onto a membrane and probed with radioactive DNA fragments described in Section 2.8. Essentially the agarose gel is UV nicked on a transilluminator and depurinated using HCl. NaOH treatment is used to induce DNA strand separation to single stranded DNA. Neutral Southern blotting is used to transfer single stranded DNA fragments to a nitrocellulose membrane, and complementary radiolabelled oligonucleotide fragments are used to analyse where individual DNA fragments sediment in the sucrose gradient (Figure 5.4, C).
A

B

0.7% TPE 25V 20hr
NaCl: 1mM 80mM
Fraction: 8 – 13 8 - 13

UV Absorption

1mM NaCl
80mM NaCl

Phenol/Chloroform + Proteinase K/Reverse crosslinking O.N

DNA Size (bp)

Fraction

5000 10000
0 8 9 10 11 12 13

80mM 1mM

C

UV

0.25M HCl

0.5M NaOH

254nm

Radiolabeled Probe
Figure 5.4. Sucrose gradient chromatin analysis methodology. Process of isolating chromatin fibers and performing compaction analyses. (A) Nuclei are prepared from cells under physiological conditions. Micrococcal nuclease partial digest allows chromatin fragments to be released from the nuclei. This chromatin solution is then spun through an isokinetic sucrose gradient. (B) The sucrose gradient is fractionated and passed through a UV spectrometer to determine the DNA content of each fraction. Each fraction undergoes a phenol/chloroform extraction to purify the DNA and run on a 0.7% TPE gel. The maxima intensity for each fraction is used to determine the DNA fragment size for each fraction and plotted to compare samples. (C) The DNA gel is treated with UV and HCl to fragment the DNA. Afterwards the gel is treated with NaOH to separate the DNA strands and single stranded DNA is transferred to a nylon membrane by Southern blotting. This membrane is then hybridized to radio labeled DNA probes for heterochromatic repeats and compared to the total DNA fragment position on the gel to assess chromatin compaction.
Gradient fractions containing chromatin fibres ranging from 2.5 kb to 12 kb were compared between wild type (WT41) and Suv39h double null (DN57) mESCs (Figure 5.5). As chromatin fibres get longer the number of possible disruptions increases, therefore, increasing the differences in sedimentation between the chromatin fibres. To facilitate a comparison in chromatin structure the sedimentation rate of chromatin fibres at 9 kb were contrasted. By reading across from 9 kb it is apparent that DN57 chromatin is sedimenting more slowly that WT41 chromatin (Figure 5.5, B). Reading the graph the other way, the bulk chromatin fragment size at Fraction 17 from WT41 cells was 8588 bp compared to 9328 bp in DN57 cells. This 8.6% discrepancy is a very surprising result that indicates that chromatin fibres in DN57 cells are on average more disrupted across the entire genome.
Figure 5.5. Effects of Suv39h1/2 loss of global large scale chromatin structure. Chromatin fibers were prepared from WT41 and DN57 nuclei preps in physiological NaCl conditions (80 mM) and resolved by sucrose sedimentation (A). DNA from sucrose gradient fractions were phenol chloroform purified and resolved on a 0.7% TPE gel at 25V for 16 hours. Left image – WT41 cells, Right image – DN57 cells. (B) Lane 2D densitometry was used to determine the position of the fraction intensity maxima. Maxima across fractions was plotted and compared between WT41 and DN57 chromatin.
The distribution of signal intensity throughout each lane represents the variation of compaction states in each fraction. The fragment size measurements are determined by the intensity maxima in each lane. In order to determine the effect of Suv39h1/2 knock out on pericentric and centric heterochromatin, fractions from the sucrose gradient were blotted and probed for major and minor satellite (Figure 5.6). Regions of constitutive heterochromatin are more compact than bulk chromatin as previously reported (Gilbert and Allan, 2001). This results in similar fragment sizes appearing in different fractions in bulk chromatin compared to heterochromatic regions. Major satellite DNA from WT41 and DN57 cells sediments into fraction 19 with 9624 bp and 10965 bp respectively. Bulk WT41 and DN57 chromatin fragments sedimented into fraction 18 with 9511 bp and 10266 bp sizes by comparison. The change in bulk chromatin sedimentation in DN57 was at similar levels to the previous experiments (7-9%) demonstrating that this change in compaction state is relatively stable. Major Satellite chromatin of similar fragment size (WT41: 9642 bp, DN57: 10965 bp) presents more disrupted sedimentation (13.7% change in 9 kb fragment size) in the Suv39h knockout system, than in bulk chromatin. This could be due to either a global de-compaction to a similar state, therefore accentuated in more compact chromatin regions; or that de-compaction is only occurring in pericentric heterochromatin.
**Figure 5.6. Effect of Suv39h1/2 loss on interphase centromere chromatin structure.** Chromatin fibers were isolated from WT41 and DN57 nuclei and resolved by sucrose sedimentation. DNA from sucrose gradient fractions were phenol chloroform purified and fractions run on 0.7% TPE gels. Gels were then southern blotted and probed for Major and Minor satellite repeats. **(A)** Images of TPE gels with chromatin fractions from WT41 and DN57 cells. Left – all (Bulk) chromatin. Right – Minor satellite chromatin from southern blot. **(B)** Fraction peak intensity maxima from WT41 and DN57 cells, of total (bulk) chromatin. **(C)** Fraction peak intensity maxima from WT41 and DN57 cells, Minor satellite probed chromatin.
To investigate if this was limited to major satellite heterochromatin, chromatin fraction for minor satellite DNA was also radiolabelled (Figure 5.6). Again bulk chromatin DNA fragment sizes in DN57 cells was 8.5% larger in fraction 17 (WT41: 8769 bp, DN57: 9523 bp). Minor satellite DNA, similar to major satellite was significantly more de-compacted than bulk in DN57 cells. WT41 cells fraction 18 DNA size was 9222 bp compared to 10353 bp in DN57 cells. This represents a 12.3% shift in DNA fragment size sedimentation of 9 kb chromatin fibres, similar to the 13.7% in major satellite DNA. This suggests that chromatin de-compaction is not limited to specific satellite repeats. Seminal studies by Kit, (1961, 1962) used CsCl gradients to quantify the satellite composition of mouse cells (10%) which has now been validated by sequencing techniques (Komissarov et al., 2011). Given the 10% nuclear abundance of satellite chromatin, if the chromatin de-compaction was limited to satellite repeats in DN57 cells, and then the global effect should be a 10th of major and minor satellite de-compaction. As this is not the evident (Bulk: 7-9%, Major: 13.7%, Minor: 12.3%) this suggests significant chromatin de-compaction at euchromatic regions.
5.3 Investigating Suv39h double null cells
global chromatin compaction

Sedimentation rate would be expected to alter if the mass of the chromatin fibre between WT41 and DN57 was different. In order to determine if the histone composition along the chromatin fibres was altered in DN57 cells, a step gradient was utilised to separate chromatin associated proteins (Figure 5.7). Chromatin fibres released after micrococcal nuclease treatment were loaded on a 50-10% sucrose step gradient described in Section 2.8.2. This forms a boundary between the two different sucrose concentrations and results in an accumulation of chromatin fibres of all sizes at the sucrose phase boundary. As unbound proteins will not sediment during centrifugation, this allows the separation of proteins bound to chromatin fibres from their soluble nuclear counterparts. Chromatin bound histone proteins were not consistently altered in the DN57 cells compared to wild type (Figure 5.7 C). This suggests that histone protein levels are not altered in DN57 chromatin fibres compared to WT41 cells.
Figure 5.7. Chromatin incorporated histone levels after Suv39h1/2 loss. Chromatin fibers released from WT41 and DN57 were purified by sucrose step gradient sedimentation. (A) 10-50% sucrose SW55 step gradient preparation and centrifugation of chromatin fibers. (B) UV absorbance traces during the step gradient fractionation process. (C) Protein lysates from chromatin containing fractions were resolved on a 10% Bis-Tris Nupage and coomassie stained to visualise histone proteins. Relative 2D densitometry was used to compare chromatin bound histone levels from WT41 and DN57 mES cells.
Chromatin accessibility to nucleases has long been used as a method used to distinguish between chromatin structures (Huebner et al., 1981). A micrococcal nuclease (MNase) timecourse digest was used to determine whether DN57 cells have altered accessibility to their DNA compared to WT41 (Figure 5.8). High molecular weight material is digested over time with MNase; DNA is cut between nucleosomes to form shorter nucleosomal fragments. DN57 cells high molecular weight material has accentuated digestion of high molecular weight DNA. Two minutes of MNase treatment reduces high molecular weight material by 18% and four minute treatment resulted in a 19% decrease compared to controls. This suggests that chromatin fibres in DN57 are more accessible to micrococcal nuclease, supporting the sucrose sedimentation experiments indicating that chromatin has a more disrupted or open configuration (Figure 5.5).
Figure 5.8. Chromatin accessibility with Suv39h1/2 loss. Nuclei were isolated from WT41 and DN57 cells, and a partial MNase digestion time course performed. Digestion was resolved on a 1% acrylamide gel. (A) Micrococcal Nuclease digest timecourse of WT41 and DN57 nuclei. 1-16 minutes. (B) Percentage of lane total pixel intensity above 4 kb for WT41 and DN57 cells, across 1-16 minutes micrococcal nuclease digest.

However as these mESCs were derived from a single clone, it was important to determine if this change in global compaction was restricted to this specific clone. Therefore, the chromatin compaction of another Suv39h double null clone was analysed. DN72 cells also presented a global change in chromatin compaction (Figure 5.9). In fraction 18, DN72 chromatin had an average DNA size of 9390 bp compared with WT41 chromatin DNA size of 8809 bp. This represents a 6.5% shift in DNA fragment size sedimentation in line with similar levels in the DN57 clone (Figure 5.5).
Figure 5.9. Bulk large scale chromatin compaction states of DN72 Suv39h1/2 clone. Chromatin fibers were purified from WT41 and DN72 nuclei and resolved by sucrose sedimentation (A) DNA from sucrose gradient fractions was phenol chloroform purified and run on 0.7% TPE gels at 25V for 16 hours. Left – DN72 cells, Right – WT41 cells. (B) Comparison of fraction peak maxima determined by 2D densitometry in WT41 and DN72 total (bulk) chromatin.
To determine if the relationship between Suv39h loss and chromatin de-compaction was direct, human Suv39h1 was reinserted into DN72 cells. Suv39h1 was cloned into the pPyCAGASIZ cassette and transfected into DN72 cells for stable expression under internal ribosome entry site (IRES) controlled zeocin resistance. To determine if Suv39h1 was stably expressed after transfections, western blots were used to quantify protein levels (Figure 5.10, A). In each DN72 clone selected, Suv39h1 was stably expressed. While exogenous human Suv39h1 was stable in these cells, expression levels were clearly below that of wild type mESCs. Despite the lower re-expression levels of Suv39h1, this was sufficient to recover H3K9me3 at chromocenters (Figure 5.10, B) and indicates that exogenous human Suv39h1 was properly recruited to chromocenters and maintained its catalytic activity.
Figure 5.10. Exogenous Suv39h1 expression and restoration of H3K9me3 at constitutive heterochromatin. (A) Western of nuclei protein lysates of Suv39h1 rescue clones from DN72 knock out cells compared to wild type and the DN72 parental cells. (B) DAPI and H3K9me3 Immunofluorescence of wild type, Suv39h1/2 knockout cells and a Suv39h1 rescue clone. 100X magnification.
In addition to H3K9me3 deposition, it was important to determine if HP1 is also recruited to chromocenters. Immunofluorescence was used to determine recruitment of HP1α to chromocenters in wild type (WT41), Suv39h double null (DN72) and exogenous Suv39h1 rescued DN72 cells (Figure 5.11). HP1α demonstrated distinct recruitment to chromocenters in the majority of Suv39h1 rescued cells. The lack of recruitment in every cell may be indicative of some cells losing the exogenous Suv39h1 construct, or its expression being slightly lower. However, the correct recruitment of HP1α in the majority of Suv39h1 rescued cells, suggest that this is sufficient for correct establishment of the Suv39h/HP1/H3K9me3 complex at constitutive heterochromatin.
Figure 5.11. Recruitment of HP1α with exogenous Suv39h1 expression. Immunofluorescence of DAPI and HP1 alpha distribution in mES cells. Wild type (WT41), parental Suv39h1/2 knockout cells (DN72) and a daughter Suv39h1 rescue clone (Clone4) were analysed after fixation. 63X magnification. Scale bar is 10 µm.
Chromatin fibre sucrose sedimentation was used to determine if the Suv39h1 re-expression in the DN72 background was sufficient to ablate the global chromatin de-compaction phenotype (Figure 5.12). At larger chromatin fragment sizes, DN72R chromatin was more compacted when compared to WT41, completely reversing the original phenotype. At fraction 17, DN72R chromatin was 9067 bp compared while WT41 chromatin was 9487 bp in size. This suggests that DN72R chromatin is 4.4% more compact at this fragment size. This result was repeated (N=2) to validate the altered global compaction state of Suv39h1 rescued mESCs.
Figure 5.12. Reversal of global decompaction with exogenous Suv39h1 expression. Chromatin fibers were isolated from WT41 and DN72R nuclei and resolved by sucrose sedimentation. (A) DNA from sucrose fractions of WT41 and Suv39h1 rescue cells from DN72 parental cells run on a 0.7% TPE gel. (B) Comparison of fragment intensity maxima from sucrose fractions of WT41 and DN72R chromatin.
Given the growing evidence of an RNA component in heterochromatin formation (Maison, Bailly, Peters, J.-P. Quivy, et al., 2002; Maison et al., 2012; Keller et al., 2013), RNA distribution was analysed by EU incorporation (Figure 5.13). Cells were treated with 1 mM EU so that the majority of EU transcripts would have EU incorporation. Cells were then washed with CSK buffer in order to remove free non-chromatin associated RNA and proteins from the nucleus and fixed with 4% PFA. A click reaction was used to covalently attach a biotin-azide to EU overnight before immunofluorescence was used to determine chromatin RNA distribution. Neither WT41, DN72, of DN72Rescue cells presented any distinct pattern to their chromatin associated RNA. However, DN72 cells did appear to have an increased intensity of EU-biotin signal.

In order to quantify chromatin bound RNAs, RNA was isolated from each cell line and analysed by dotblot (Figure 5.14). Similar to EU immunofluorescence experiments; each cell line was incubated with 1 mM EU overnight to incorporate EU into RNA. Cells were washed to remove non-chromatin bound RNA, before being trypsinised and the nuclei isolated, before further NB.B buffer incubation to remove the nuclear membrane. The Biotin-azide was then clicked onto the RNA overnight at room temperature before samples were normalised to the DNA concentration and spotted onto a nitrocellulose membrane. Avidin-HRP incubation was then used to quantify EU-RNA concentration. DN72 cells showed a 25% increase in chromatin associated RNA when compared to WT41. Additionally, the Suv39h1 rescue cell line had a further 10% reduction in chromatin associated RNA. These results correlate with the chromatin compaction states between the cell lines and suggest that Suv39h/HP1/H3K9me3 heterochromatin recruitment directly affects the global levels of chromatin associated RNA.
and chromatin compaction states. While this data demonstrates an altered level of chromatin associated RNA, this does not explain the full de-compaction of chromatin fibres after sucrose sedimentation, as these fibres have been prepared in the presence of RNase and MNase. However, this provides further evidence towards a regulatory role of chromatin associated RNA in heterochromatin formation and beyond in the genome.

Figure 5.13. Suv39h1/2 loss/rescue and chromatin associated RNA. mES cells treated with 1 mM EU O.N, then washed in CSK buffer and fixed in 4% PFA for 10 minutes, further washed in 0.5% Triton-X for 10 minutes, before click reaction with an azide fluorophore and DAPI stained for 3 minutes at room temperature. Chromatin associated RNA was imaged from wild type (WT41), Suv39h1/2 double null (DN72) and human suv39h1 rescue (Clone 4, 5) mouse ES cells. 100X magnification.
Figure 5.14. Effect of Suv39h methyltransferase expression on global chromatin associated RNA levels. (A) mES cells treated with 1 mM EU O.N before 3 x CSK washes for 5 minutes and nuclei isolation. RNA was prepared using Qiagen RNeasy kit instructions, and a biotinylated azide added via click chemistry. Samples were spotted on nitrocellulose membranes and incubated with avidin-HRP for detection. (B) 2D densitometry of chromatin bound RNA dot blot for wild type, Suv39h1/2 knockout DN72 cells, and a DN72 Suv39h1 rescue clone (clone 4).
5.4 Click chemistry DNA fixation: method optimisation

While the chromatin compaction studies, such as sucrose gradient sedimentation, are able to distinguish between chromatin fibres with different structures, but whether these are representative of *in vivo* chromatin is still not clear. As previously mentioned, the inevitable digestion of potential chromatin-associated RNAs by MNase could alter chromatin compaction (Maison, Bailly, Peters, J.-P. Quivy, *et al.*, 2002), and as the chromatin fibres released are not fixed, any torsional forces (such as DNA supercoiling) within the chromatin fibre will be lost. Finally, the process of releasing soluble chromatin fibres may directly alter their structure, despite physiological salt conditions being used, as this environment cannot compensate for the local hydrophobicity of the surrounding liquid state that is caused by protein and RNA concentrations in the nucleus (Larson *et al.*, 2017).

By comparison, altered salt conditions affect the ionic interactions between nucleosomes, and therefore can significantly affect chromatin compaction (*Figure 5.15*). Release of 3T3 chromatin into 1 mM NaCl compared to 80 mM NaCl (physiological levels) and subsequent characterisation of chromatin structure by sucrose gradient sedimentation shows that chromatin in low salt has a significantly more open or disrupted chromatin fibre structure.
Figure 5.15. Decompaction of unfixed chromatin in low NaCl conditions. Chromatin fibers were isolated from mouse 3T3 cells and resolved by sucrose sedimentation in physiological (80 mM) and low (1 mM) NaCl conditions. (A) purified 3T3 cell DNA from 80 mM and 1 mM sucrose gradient fractions resolved by 0.7% TPE gel electrophoresis. (B) Comparison of fraction fragment intensity maxima identified by 2D densitometry of chromatin in physiological and low NaCl conditions.
Although formaldehyde is a commonly used and fast fixation method it is very indiscriminate and after cross-linking MNase can no longer efficiently digest chromatin and soluble fibres cannot be released (Figure 5.16, A-B). Treatment of 1% formaldehyde prevents any MNase digestion within 16 minutes, while 1% FA treatment after MNase digestion requires SDS addition to release chromatin fibres. Unfortunately, detergent treatment also disrupts chromatin fibre compaction and chromatin no longer sediments well in sucrose gradients indicative of heterogeneous and disorganised fibres. This could suggest that chromatin fibres have dramatically more diverse structures within the genome, or more likely that 1% FA treatment crosslinks between chromatin fibres of different sizes. This would result in shorter chromatin fibres ‘piggybacking’ on the larger chromatin fibres altering the sedimentation rate and fibre size distribution.
Figure 5.16. Difficulties of fixed chromatin preparation and click chemistry. Chromatin fibre release under formaldehyde (FA) fixed conditions. (A) Micrococcal digest of nuclei FA 1% fixed for 10 minutes RT. (B) DNA from sucrose gradient fractions with nuclei FA 1% fixation after micrococcal digest, treated with 0.5% SDS to release chromatin fibers. (C) DNA analogue EdU incorporation and alkyne click reaction (D) PEG-Bis-azide (E) Picolyl-bis-azide linker (F) 3-azido-7-hydroxycoumarin
Instead of using formaldehyde as an indiscriminate cross-linker we investigated the idea of specifically crosslinking DNA as it might still allow MNase accessibility and maintain chromatin fibres’ flexibility in order to be released from the nucleus (Figure 5.16, C). Click chemistry could be used as a method to cross-link DNA-DNA due to its highly specific reaction conditions. In a click reaction an alkyne group is covalently attached to an azide group forming a triazole (Jiang et al., 2014). A copper ion initially attacks the alkyne group weakening the triple bond. This allows another copper ion to associate with the alkyne group. The positive charge of the second copper ion then attacks the base nitrogen atom of a nearby azide group. Through electron pushing this causes the electron pair in the alkyne initially interacting with the second copper ion to switch to third nitrogen in the azide group resulting in the covalent bonding between the two groups (Figure 5.17). This click reaction has already been extensively used in vitro to covalently attach molecules to nucleic acids (Kumar et al., 2007; Gramlich et al., 2008).

Additionally this click reaction has been used to crosslink fluorophores to cellular membranes in vivo but has not been presented within the nucleus of live cells (Uttamapinant et al., 2012; Jiang et al., 2014). As free metals are toxic in cells, the free copper ion cellular availability inside cells must be highly regulated. Copper ions are therefore added to cells in the form of CuSO₄ to reduce initial toxicity. In order to release copper ions, sodium ascorbate is used as a weak reduction catalyst. To further reduce free copper ion toxicity a sequestering partner such as THPTA or TBTA is added and this enabled fluorophore labelling within zebrafish (Clavadetscher et al., 2016).
Figure 5.17. Click Chemistry azide-alkyne reaction. Mechanistic rational for copper assisted akyne-azide cycloaddition (CuAAC). Diagram adapted from Jiang, et al. (2014)

However, in order to fix chromatin fibre structure in cells the density of cross-links must be high and the reaction speed fast enough to prevent the likelihood of trans-fibre crosslinking. In order to determine if nucleotide analogues conjugated to an alkyne could be incorporated at a high enough density to facilitate chromatin fixation, cells were treated with EdU and EdC at 20 µM for up to 72 hours (Figure 5.18, A). The DNA was purified, digested to mononucleotides, analysed by HPLC and quantified using UV spectroscopy. The retention times and absorption profiles of the uridine analogues were determined via input standards. Under these conditions both EdC and EdU were detectable as being incorporated into the genome. In our hands the EdU derivative F-ara-EdU incorporation was barely detectable (data not shown) despite being sufficient for click based immunofluorescence (Neef and Luedtke, 2011). F-ara-EdU has been proposed as a less toxic tool
than EdU and BrdU however this may be due to significantly reduced incorporation rates during replication. F-ara-EdU was therefore rejected as a potential analogue for click-mediated chromatin fixation.

EdU and EdC had similar incorporation rates to each other with over 15% of all nucleotide incorporated after 72 hours. EdC incorporation plateaued between 24 and 48 hours, suggesting that replication rate after EdC incorporation was affected (Figure 5.18, A). To determine how stable analogue incorporation was and if they were excised by the repair process, cells were treated with 20 µM EdC or EdU for 72 hours, washed out and then a further 48 hours without analogue incorporation (Figure 5.18, B). Both EdU and EdC presented a partial decrease in analogue incorporation after free analogue nucleotides were removed. This would be expected, as future replication cycles would dilute the analogue nuclear concentration. The relatively stable incorporation of EdU and EdC analogues suggest that both would be suitable candidates for a click-based fixation method.

To determine if nucleotide analogue incorporation affected the ratio of endogenous nucleotide (A-T, C-G), Nucleotide ratios to dG were compared over dose ranges of EdU and EdC (Figure 5.18, C-D). Given that EdU and EdC are thymidine and cytidine analogues respectively, the levels of adenine and guanine should not be affected by analogue incorporation. With both EdU and EdC treatment: dG, dA, and methyl dC levels were not affected. Both EdU and EdC incorporation levels were inversely correlated with thymidine levels, suggesting that both are incorporated in place of thymidine. The fact that adenine and guanine ratios were unaffected by EdC treatment also implies that when EdC is incorporated instead of a thymidine, it is still paired correctly with an adenine nucleotide.
Figure 5.18. Alkyne nucleotide analogue incorporation. 3T3 cells incubated with DNA analogues and their DNA digested to mononucleotides for HPLC-UV analysis. (A) Percentage of DNA analogues [EdU and EdC] incorporation into mESc DNA at 20 µM over time. (B) DNA analogue incorporation density at 20 µM for 72 hours and after an additional 48 hours without DNA analogue treatment. (C) Individual mononucleotide ratios to dG after EdU dose range exposure (D) Individual nucleotide ratios to dG after EdC dose range exposure.
To determine if EdC was incorporated after being modified, the spectrometry profiles of incorporated EdC was compared to EdU and EdC standards. Incorporated nucleotides will always have a slightly altered spectrometry profile however due to the attached phosphate group required for incorporation into the DNA backbone. Despite this the EdC profile absorption peaks were more aligned with EdU than EdC standards (Figure 5.19, A). To further understand how EdC is modified when it is incorporated, EdC and EdU treated cells’ nucleotides were analysed by HPLC-MS-MS (Figure 5.19, B-C). Peaks for the predicted EdU-monophosphate mass were detected in both EdU and EdC treated cells. The MS/MS fragmentation of these peaks revealed identical profiles for EdU and EdC. Together these data demonstrate that EdC is deaminated to an EdU molecule before its incorporation into DNA. This is an important finding as many studies (Petryk et al., 2016) use EdC instead of EdU to control for sequence bias.
Figure 5.19. DNA analogues EdU and EdC UV spectra and MS fragmentation profiles. 3T3 cells were incubated with 20 µM DNA analogues for 24 hours before their DNA was digested to mononucleosomes. Samples were analysed by HPLC-UV or Mass spectrometry and compared to purified standards. A) DNA analogue UV spectrometer intensity profiles resolved by HPLC retention time. Comparison between incorporated EdC and the purified standards for EdU and EdC. B) Retention time profiles of incorporated DNA analogues of predicted EdUMP m/z by HPLC-MS C) MS/MS fragmentation profiles of incorporated EdC and EdU DNA analogues.
Figure 5.20. Azide-alkyne *in vitro* plasmid click reaction and clicked coumarin-azide fluorescence. **A)** *In vitro* click reaction of EdU incorporated Recon2 plasmid digested with Xho1. E.coli with Recon2 plasmid were incubated with 20µM EdU for 3 hours at 37°C. Plasmid was purified using the Qiagen mini prep protocol and digested O.N with Xho1 at 37°C. Click reaction was then added at RT: CuSO4 0.5mM, THPTA 0.25mM, NaAsc 250mM, 100mM PEG-bis-azide (5000) **B)** 10X 400ms 488nm of mouse 3T3 cells incubated with 20µM EdU O.N before click reaction added for 60 minutes: CuSO4 0.5mM, THPTA 0.25mM, NaAsc 250mM, 200mM Coumarin-azide.
As the incorporation of exotic nucleotides into DNA is normally unwanted during replication, the effect of EdU/EdC incorporation on the cell cycle was investigated (Figure 5.21). Propidium Iodide staining and FACS analysis showed that both a 24 hour 20 μ M EdU and EdC treatment increased the number of cells in S-phase. Significantly though, cell numbers in G2 that have exited S-phase was elevated in EdC but not EdU treated cells. This suggests that the deamination process required for EdC incorporation resulted in replication stress causing cellular arrest. By contrast, EdU treatment increased the time in S-phase but did not result in stalling of cells in G2, nor a significant increase in polyploidy. Together this evidence indicates that EdU is the analogue of choice for incorporating an alkyne tag into genomic DNA for click chemistry based cross-linking.

In order to optimise the click chemistry reaction in vivo a 3-azido-7-hydroxycoumarin compound was used (Figure 5.16 D-F). 7-hydroxycoumarin is a blue wavelength fluorophore between 404 and 480 nm and its fluorescence is quenched in the presence of the 3-azido sidechain. This quenching by the side group is however lost when the azide reacts with an alkyne to form a triazole (Gierlich et al., 2006) and results in an order of magnitude (15x) increase in fluorescence in the clicked coumarin form (Li et al., 2010). The use of a coumarin-azide fluorophore therefore allows the monitoring of click reaction kinetics in live cells for optimisation (Figure 5.20, B).
Figure 5.21. Effect of nucleotide analogue incorporation on the cell cycle. 3T3 cells treated with 20 μM of DNA analogues for 24 hours, ethanol fixed and propidium iodide (PI) stained for flow cytometry. (A) PI stain cell cycle analysis after EdU or EdC 20 μM treatment of mES cells for 24 hours and controls. Histogram plots of PI fluorescent intensity. (B) Gated cell cycle stages ratios from flow cytometry PI plots, analysed by FlowJo.
In order to determine if the coumarin-azide was detectable in cells, 3T3 cells were treated with EdU for 24 hours and were fixed with 1% paraformaldehyde. After fixation cells were incubated with coumarin-azide in a click reaction (Figure 5.22) and a dose dependent incorporation was observed. In live 3T3 cells however an increase in coumarin fluorescence was only detectable at 500 µM CuSO₄. This suggests that live cells actively inhibit the click reaction mechanism, which is possibly due to native cellular copper chelators evicting free copper ions efflux transporters (Petris, 2004).
Figure 5.22. Coumarin-azide click reaction kinetics in live cells. 3T3 cells were incubated with 20 µM EdU for 24 hours. (A) Intensity of coumarin-azide fluorescence in 3T3 cells 1% formaldehyde fixed before click reaction. 3T3 cells were washed with CSK buffer prior to fixation. Click reaction: ΔµM CuSO₄, 10 mM NaAsc, 200 mM coumarin-azide added to PBS. (B) Intensity of coumarin-azide fluorescence in 3T3 cells after click reaction in the presence of PBS: ΔµM CuSO₄, 10 mM NaAsc, and 200 µM Coumarin-azide.
For click reactions to be conducive to fixing chromatin structure it must be efficient, fast, accurate, and non-toxic to the host cell. To achieve this the metal, chelating agent, and catalyst need to be optimised. Different metals and concentrations were first tested on 3T3 cells incubated with 20 µM EdU for 24 hours (Figure 5.23). In addition to CuSO₄; Ruthenium, MgCl₂ and CuBr₂ have previously been used as metal agents in click reactions and their efficacy was analysed (Pérez-Balderas et al., 2003; Boren et al., 2008). Cells were incubated with 250 mM coumarin-azide, 10 mM sodium ascorbate in the presence of each metal salt. Both ruthenium and magnesium chloride had delayed onset of coumarin fluorescence and did not present the expected sigmoidal reaction kinetics. Contrastingly CuSO₄ and CuBr₂ demonstrated dose dependent increases in fluorescence kinetics. At lower concentrations this dose dependent effect was more sensitive in CuSO₄ suggesting that it may be the optimal click reaction catalyst within this setting.

To determine if a chelating agent would accelerate or slow the reaction rate, a dose range of THPTA was added to 200 µM CuSO₄, 200 mM Coumarin-azide, and 10 mM sodium ascorbate (Figure 5.24). Increasing the THPTA concentration increased the reaction speed up to 100 µM, after which higher concentrations resulted in decreased reaction kinetics. This suggests that the chelating agent’s ability to prevent free copper ion excretion increases the reaction speed up to a 1:2 ratio with CuSO₄. As the chelating agent’s concentration increases above this ratio, the increased kinetics from preventing excretion is offset by the sequestering of the free copper ions from catalysing the click reaction. While other chelating agents were tested (TBTA, BTTP) THPTA presented the fastest click reaction kinetics (data not shown).
Figure 5.23. Live cell click chemistry metal catalyst optimisation. Comparison of different metals as click reaction catalyst. 3T3 cells were incubated with 20 µM EdU for 24 hours prior to click reaction. Click reaction conditions: Δ µM Metal, 250 mM Coumarin-azide, 10 mM NaAsc. And normal mESc growth media. Metals tested were; (A) CuSO₄, (B) Ruthenium, (C) MgCl₂, and (D) CuBr₂.
Figure 5.24. Live cell click chemistry metal chelator optimisation: chelating agent. Optimization of chelating agent THPTA concentration. 3T3 cells were incubated with 20 µM EdU for 24 hours prior to click reaction. Click reaction conditions: 200 µM CuSO₄, Δ µM THPTA, 200 mM Coumarin-azide, 10 mM NaAsc.
For free copper to be chelated by THPTA and used in a click reaction, it must first be reduced from the CuSO₄ source. Sodium ascorbate (NaAsc) and ascorbic acid have traditionally been used as the reducing agent for free copper ions (Himo et al., 2005). As NaAsc is less toxic than ascorbic acid a dose range of NaAsc was added to 500 µM CuSO₄, 200 mM coumarin-azide, 250 µM THPTA (Figure 5.25). NaAsc increased the click reaction kinetics in a dose dependent manner up to 250 mM. Concentrations above 250 mM of NaAsc did not present any increase in reaction speed. This implies that after a NaAsc:CuSO₄ ratio of 500:1, the reduction of CuSO₄ to free copper ions is not the limiting factor to the reaction speed. However, under these conditions the click reaction was complete in 5 hours. To try and increase the reaction speed further, lipofectin or Triton-X100 was used to disrupt the cell membrane to prevent copper ion excretion. However, Triton-X100 and lipofectin conditions had no benefit to click reaction kinetics (Figure 5.26).

**Sodium Ascorbate**

![Figure 5.25. Live cell click chemistry reducing agent optimisation: reduction agent. Optimization of catalyst NaAsc concentration. 3T3 cells were incubated with 20 µM EdU for 24 hours prior to click reaction. Click reaction conditions: 500 µM CuSO₄, Δ mM NaAsc, 200 mM coumarin-azide, 250 µM THPTA.](image)
Figure 5.26. Live cell click chemistry, cell membrane disruption optimisation. A) Optimization of click reagent transfection. 3T3 cells incubated with 20µM EdU for 24 hours before the addition of 250mM coumarin-azide, 250mM NaAsc, and 250µM THPTA. 500µM CuSO4 addition after 6 hours to start the click reaction. B) Optimisation of detergent addition to EdU click reaction. Δ% Triton-X added to click reaction mix: 250mM Coumarin-azide, 250mM NaAsc, 250µM THPTA, and 500µM CuSO4.
This timeframe for reaction completion (5 hours) is biologically relevant due to the incorporation levels of EdU (**Figure 5.18, A**). As each nucleosome is wrapped around 147 bp of DNA, and mouse cells have a GC content of 51.24%, there should be ~70 AT nucleotide pairs on each nucleosome (Romiguier et al., 2010). With 20 μM EdU treatment for 24 hours, 7% of all nucleotides are EdU molecules, representing 14.35% of all thymidines. Together these data indicate that each nucleosome will have 7-14 alkyne tags.

To maintain chromatin structure, crosslinking each nucleosome to its adjacent neighbours once should be sufficient to maintain fibre structure. Therefore only 20% of the total reaction potential (1 hour) should be needed to fix chromatin structure. This reaction time can theoretically be reduced further by the use of a picoyl-azide crosslinker (**Figure 5.16, E**) which should decrease the reaction time by an order of magnitude (Uttamapinant et al., 2012; Jiang et al., 2014).

Having optimised the click reaction conditions, the use of a bis-azide crosslinker to covalently bond two DNA fragment was investigated *in vitro* (**Figure 5.20**). E.coli cells transformed with a pUC57-Recon2 plasmid were grown overnight. 20 μM EdU was added for 3 hours before cells were lysed and plasmids extracted and purified. 3 hour 20 μM EdU treatment was previously determined to be sufficient for >10% EdU incorporation by HPLC-UV spectrometry (data not shown). Two DNA fragments were prepared by digesting the EdU containing pUC57-Recon2 plasmid with XhoI restriction endonuclease. Optimised click reaction conditions (24 hours of 20 μM EdU incubation, 250 mM NaAsc, 250 μM THPTA, and 500 μM CuSO₄) with an bis-azide PEG-linker (average M.W 5000 Da) were added to the digested Recon2 plasmid for 1 – 8 hours. Within 1 hour interstrand crosslinking had occurred resulting in two additional DNA bands and after 4
hours intrastrand crosslinking resulted in smearing of the DNA bands. This suggests that a click reaction can crosslink between naked DNA strands in vitro.

To establish if these click reaction conditions would be sufficient to fix chromatin fibre structures in live cells, sedimentation rates of clicked chromatin fibres were compared in sucrose gradients with 1 mM or 80 mM salt (Figure 5.27). 3T3 cells were incubated with 20 μ M EdU and 100 mM PEG-bis-azide for 24 hours. Cells were then crosslinked via a click reaction with 500 μ M CuSO₄, 250 μ M THPTA, and 250 mM NaAsc for 1 hour. Chromatin fibres were digested with MNase and then released as presented in figure 5.4. The clicked chromatin fibres were loaded onto isokinetic sucrose gradients in TEEP buffer with either 80 mM NaCl or 1 mM NaCl buffer. As 1 mM NaCl causes 3T3 chromatin fibres to de-compact substantially (Figure 5.15), if click crosslinking had fixed the chromatin structure the sedimentation rate should have been the same in 80 mM or 1 mM NaCl gradients.

Unfortunately, 3T3 click fixed chromatin unfolded in 1 mM NaCl, suggesting that cross-linking was not sufficient to stabilise the chromatin structure (Figure 5.27). In fraction 13, 80mM salt conditions had an average DNA fragment size of 5213 bp compared to 6728 bp in 1 mM NaCl conditions. This represents a 29% shift in DNA fragment size sedimenting in fraction 13. This suggests a significantly slower sedimentation rate in 1mM NaCl conditions. However, unfixed 3T3 chromatin sedimenting in fraction 12 in 80 mM NaCl buffer had an average fragment size of 5176 bp whilst in 1 mM NaCl the chromatin sedimenting in fraction 12 was 9595 bp (Figure 5.15). Therefore unfixed chromatin of similar fragment sizes to fixed chromatin presents an
85% shift in DNA fragment size. This suggests that a click-based cross-linking approach can fix chromatin fibres to an extent but cannot prevent other physiological properties from partially altering the chromatin compaction state.

Figure 5.27. Azide-alkyne click reaction link chromatin stability in low NaCl conditions. Azide-alkyne clicked chromatin fibers were released from 3T3 nuclei into physiological (80 mM) and low (1 mM) NaCl conditions and resolved by sucrose sedimentation. 3T3 mouse fibroblast cells incubated with EdU 20 µM EdU and 100 mM PEG-Bis-azide (5000 MW) for 24 hours prior to click reaction. Click conditions: CuSO$_4$ 0.5 mM, THPTA 0.25 mM, 250 mM NaAsc for 1 hour (A) DNA from 3T3 cell clicked chromatin sucrose fractions, at 80 mM or 1 mM NaCl buffer conditions. Fractions 10-18 run on a 0.7% TPE gel. (B) Comparison of fragment intensity maxima from fractions in each lane of 1 mM or 80 mM treated clicked chromatin.
5.5 Discussion

5.5.1 H3K9me3 and chromatin compaction

Suv39h1 and Suv39h2 are the main methyltransferases responsible for H3K9me3 (Hall et al., 2002). H3K9me3 is specifically limited to regions of constitutive heterochromatin including pericentric chromatin, telomeres and retrotransposons across the genome (Arnoult, Van Beneden and Decottignies, 2012; Bulut-Karslioglu et al., 2014). This has led to a long-standing hypothesis that H3K9me3 has an important functional role in heterochromatin formation. Loss of Suv39h enzymes results in the loss of Suv39h, H3K9me3, H4K20me3, DNA methylation and HP1 proteins from constitutive heterochromatin regions (Peters et al., 2001). However DNA methylation levels at heterochromatin have been shown to have no functional role in regulating heterochromatin compaction (Gilbert, Thomson, Boyle, Allan, Ramsahoye and Wendy A Bickmore, 2007). Given that Suv39h double null mice are viable, still form nuclear chromocenters, and only have a mild genome instability phenotype, the function of H3K9me3 in having a causal role in chromatin compaction remains unclear (Peters et al., 2001). Furthermore, no epigenetic modifications have been directly demonstrated to have a causal role in chromatin fibre compaction, even histone acetylation.
Initial experiments shown here were focused on confirming that Suv39h double null mESC cell line presented the same profile of epigenetic marks as previously reported. H3K9me3, H4K20me3 and HP1α were all shown to be lost from chromocenters in Suv39h double null ES cells (Figure 5.3, 5.10). This supports the current view that Suv39h HMTs’ specificity to H3K9me3 deposition results in the recruitment of Suv420 HMTs and HP1 proteins to constitutive heterochromatic domains (Schotta et al., 2004).

In addition to H3K9me3 nuclear distribution, total levels of H3K9me2 and H3K9me3 were significantly reduced along with its pericentric heterochromatin recognition partners HP1α and HP1β (Figure 5.2). HP1γ levels were increased in the double null cells. However HP1γ is thought to function exclusively in euchromatin and therefore does not function at the pericentromere outside of S-phase (Minc, Courvalin and Buendia, 2000). Interestingly, H3K27me3 levels were significantly elevated suggesting an expanded regulatory role for polycomb silencing in Suv39h double null cells. Recent studies have begun to implicate PRC2 function with DNA methylation and HP1 chromatin stabilisation. Reddington et al., (2013) demonstrated that a loss of DNA hypomethylation led to a global redistribution of DNA methylation, while comprehensive studies by Manzo et al., (2017) described the detailed function of H3K27me3 and H3K4me3 in excluding DNMT3 isoforms from their bivalent chromatin domains but recruiting DNMT3A1 to their periphery.
While H3K27me3 is not found at pericentric regions, the principle of mutual exclusivity with H3K9me3 has been demonstrated to be false. Mass spectrometry of H3K27me2 and H3K27me3 containing nucleosomes from HeLa, mESC, and MEF cells consistently had H3K9me2/me3 and H4K20me2 marks (Voigt et al., 2012). Additionally, chromatin immunoprecipitation experiments have found co-occupation of H3K9me3 and H3K27me3 at repetitive elements, telomeres, and bivalent promoters (Zeng et al., 2009; Hawkins et al., 2010; Arnoult, Van Beneden and Decottignies, 2012). Further studies have demonstrated that PRC2 subunits Ezh2 and Suz12 are required for HP1α recruitment to heterochromatin but not H3K9me3 deposition (Boros et al., 2014). In the absence of H3K9me3, DNA methylation and HP1 heterochromatin recruitment, a compensatory increase in PRC2 mediated H3K27me3 deposition would not be unexpected and may cause the relatively mild mouse model phenotype (Peters et al., 2001).

Having demonstrated that DN57 cells lacked H3K9me3, HP1α, and H4K20me3 at chromocenters, the effect of this altered epigenetic state on chromatin compaction was investigated. Unlike DNA methylation (Gilbert, Thomson, Boyle, Allan, Ramsahoye and Wendy A Bickmore, 2007), loss of the H3K9me3/HP1/Suv39h complex from heterochromatin results in the de-compaction of pericentric (major satellite) and centromeric (minor satellite) chromatin (Figure 5.6). However, this chromatin de-compaction was not limited to pericentric and centromeric domains, as Suv39h double null cells presented global de-compaction of chromatin fibres when compared to wild type (Figure 5.5). This altered the sedimentation rate by 7-9% for 9 kb DNA fragments compared to 13.7% for Major satellite and 12.3% for minor satellite chromatin fibres of similar size.
As <10% of the mouse genome is made up of major and minor repetitive elements, if chromocenter de-compaction was solely responsible for the global effect, the de-compaction effect would be expected at a 10th of the effect at pericentric regions (Kit, 1962; Komissarov et al., 2011). This therefore suggests changes in chromatin compaction beyond the normally H3K9me3 marked chromocenters. A possible mechanism for the global de-compaction may come from interspersed repetitive elements. Around 38% of the mouse genome is composed of interspersed repetitive elements, with 19% of the genome being LINEs, 8% SINEs, and 10% LTR elements (Waterston et al., 2002). Recent studies have demonstrated Suv39h mediated H3K9me3 at ~5% of repetitive elements in mESCs (Bulut-Karslioglu et al., 2014). However, in order for repetitive elements to result in the global chromatin de-compaction, every element would need to be regulated by Suv39h, which has not been demonstrated. Together this suggests that at least some of the global chromatin de-compaction is likely due to changes in chromatin structure at genic regions.
To determine if this effect was due to clonal variation, the chromatin compaction state of an additional Suv39h double null clone (DN72) was investigated (Figure 5.9). DN72 also demonstrated a slower sedimentation rate (6.5% change in DNA fragment size at 9 kb) indicative of global de-compaction of chromatin fibres albeit slightly lower than DN57 cells (7-9%). To determine if this was directly linked to Suv39h activity, exogenous human Suv39h1 was transfected via a pPyGAGASIZ cassette. This resulted in stable expression of human Suv39h1 with the Suv39h double null mESC background, although at lower levels than endogenous Suv39h1/2 in wild type cells (Figure 5.10). The expression of human Suv39h1 was subsequently sufficient to restore H3K9me3 deposition at chromocenters and HP1α recruitment (Figure 5.11). Together this suggests that human Suv39h1 expression was sufficient to ablate the endogenous Suv39h double null phenotype.

When chromatin fibres from DN72R cells (expressing exogenous human Suv39h1) were compared to wild type chromatin, the original chromatin de-compaction phenotype was completely reversed. DN72R chromatin fibres were comparatively 4.4% smaller than WT41 suggesting a more compact chromatin fragment for a 9 kb DNA fragment size (Figure 5.12). This demonstrates for the first time a direct relationship between a histone modification deposition and chromatin fibre compaction. The increased compaction beyond wild type could be due to slightly differing functionality of human Suv39h1 compared to mouse Suv39h1. Alternatively, regulatory changes induced by Suv39h1/2 KO results are not completely reversed by human Suv39h1 expression and in combination with exogenous Suv39h1 expression contribute to chromatin compaction.
Given the growing body of evidence suggesting an RNA component to heterochromatin regulation (Arnoult, Van Beneden and Decottignies, 2012; Maison et al., 2016; Shirai et al., 2017), the levels and distribution of chromatin associated RNA was investigated (Figures 5.13, 5.14). Loss of Suv39h HMTs in DN72 clearly led to an increase in chromatin associated RNAs that was reversed with exogenous human Suv39h1 expression. The major question from this result is whether Suv39h/H3K9me3/HP1α recruitment mediates the RNA’s chromatin association, or whether Suv39/H3K9me3/HP1 supresses the transcription of ncRNAs from the underlying DNA. This remains a pertinent question yet to be answered in understanding heterochromatin regulation. While Suv39h and HP1 proteins clearly interact with RNAs, the global chromatin de-compaction cannot be completely attributed to altered Suv39h-HP1 recruitment (Meehan, Kao and Pennings, 2003; Bulut-Karslioglu et al., 2014; Maison et al., 2016). Therefore the regulation of underlying ncRNAs by Suv39h H3K9me3 deposition would project changes in chromatin associated RNAs beyond regions bound by Suv39h-HP1 complexes. This would be more representative of the global chromatin de-compaction observed in these experiments.
5.5.2 Sucrose sedimentation and click-fixation

The validity of analysing unfixed chromatin fibres remains a debated question, particularly in relating the sedimentation properties of chromatin in vitro to an in vivo structure. Formaldehyde fixation is known to induce aggregate formation that can result in large biases in chromatin immunoprecipitation (ChIP) experiments (Baranello et al., 2016). This aggregate formation prevents MNase catalytic activity and chromatin fibre release (Figure 5.16, A). Furthermore, in the same way as GFP can be artificially recruited by formaldehyde in ChIP experiments, the dynamic properties of the chromatin fibre could result in artificial formaldehyde induced chromatin compaction.

However, if the chromatin fibre is unfixed the process of micrococcal nuclease digestion will release the torsional stress in the fibre. Additionally the process of releasing chromatin fibres into solution could alter the chromatin state. Whether formaldehyde fixation or native methods are representative of the true physiological chromatin state in vivo remains debated and has led to the development of native ChIP and native conformation capture methods (i4C) (Dorbic and Wittig, 1986; Thorne, Myers and Hebbes, 2004; Brant et al., 2016).

While formaldehyde fixation is possible after MNase treatment, the nuclei must be treated with SDS in order to extract the fixed chromatin fibres (Figure 5.16, B). This suggests that a degree of fibre flexibility in the chromatin fibre is required for chromatin release. However, the use of detergents to disrupt ionic bonding and denaturing proteins could alter
chromatin compaction to a greater extent than no fixation. Additionally, the smearing in SDS formaldehyde fixed fractions is indicative of interstrand crosslinks from a large chromatin fragment to a small fragment. This significantly disrupts the resolution of the experiment and induces an additional bias (Figure 5.16, B).

To try and circumvent the negative drawbacks of formaldehyde fixation and native chromatin fibre sucrose sedimentation, the use of copper assisted cycloaddition chemistry to covalently crosslink only DNA was investigated. By only creating DNA:DNA crosslinks this should maintain some fibre flexibility while preventing aggregation. Additionally this method will allow fixation before MNase digestion and therefore before chromatin RNAs are affected (Huebner et al., 1981; Maison, Bailly, Peters, J.-P. Quivy, et al., 2002).

Preliminary studies demonstrated that thymidine-alkyne tags could be incorporated into DNA at high density without inducing cell cycle arrest (Figures 5.18, 5.21). The discovery that EdC is deaminated before DNA incorporation demonstrates a bias in DNA incorporation (Figures 5.18, D, 5.20). At a 10 nm chromatin fibre scale, linker regions are enriched for adenine-thymidine nucleotides, suggesting that more alkyne tags will be between nucleosomes (Trifonov and Sussman, 1980; Cui and Zhurkin, 2009). This may affect the accessibility of the alkyne tag while affecting its ability to fix the chromatin conformation. Furthermore, at large scale chromatin structure, euchromatic regions are enriched for AT sequences compared to heterochromatin (Dekker, 2007). This suggests that the distribution of alkyne tags will be skewed to euchromatic regions. Therefore the use of EdC as a single agent or in combination with EdU will not increase coverage at heterochromatin domains when compared to euchromatin.
A high incorporation rate is therefore required to assure genome coverage with multiple alkyne tags per histone. A nucleosome has ~147 bp of DNA wrapped around it, of which 58.76% on average (SD: 7.85%) in mice would be A-T base pairs (Romiguier et al., 2010). Therefore, each mouse nucleosome will have between 49 and 95 thymidine nucleotides across both DNA strands. With 20 µM EdU treatment, EdU makes up 7% of all incorporated nucleotides, corresponding to 7-14 EdU molecules per nucleosome (Figure 5.18, A). As copper assisted cycloaddition results in a triazole mediated bond, one crosslink with each adjacent nucleosome should be sufficient to maintain the chromatin fibre structure.

Although nuclei are porous, allowing small molecules to enter by diffusion, cell membranes contain multiple efflux transporters to remove metals and toxins (Petris, 2004). These cellular processes limit the click reaction speed, requiring optimisation for fast and efficient reactions (Figure 5.22). Once optimised completion of cellular click reactions of incorporated EdU occurred within 5 hours (Figures 5.25, 5.26). This would allow between 1-3 click reactions per nucleosome per hour with a standard azide.

Further chemical studies have indicated that this reaction speed can be further enhanced from the addition of a picoyl group inside the azide group. Azide picoyl groups chelate with the first copper ion that attacks the alkyne group reducing the partial molar free energy needed 25 fold for the click reaction (Uttamapinant et al., 2012). The can be further reduced by reversing the amide group on the opposite side of the picoyl group from the azide (Figure 5.16, E). This makes the amide electron donating and further increases the reaction speed 2-3 fold. The subsequent 50 fold reduction of reaction time via a picoyl-azide would allow reaction completion in 10-12
minutes in live cells. Although a bis-picoyl-PEG crosslinker was designed, this was not synthesised within the timeframe of this study.

Instead, a PEG linker (MW, 5 kDa) with two azide groups was used to crosslink DNA fragments (Figure 5.16, D). Using in vitro naked DNA, interstrand crosslinks were visible after 1 hour (Figure 5.20). Following these positive results the ability of a 1-hour click reaction to fix EdU containing chromatin fibres was investigated and analysed by sucrose sedimentation (Figure 5.27). The ionic conditions of the chromatin solution, has previously been shown to dramatically alter the chromatin compaction state with low NaCl concentrations associated with de-compaction (Thoma, et al., 1979). To determine if a 1-hour click reaction could fix chromatin structure in live cells, Click-fixed chromatin was analysed in physiological (80 mM) and low (1 mM) NaCl conditions (Figure 5.27). Unfortunately, in 1 mM NaCl click-fixed chromatin demonstrated significant de-compaction compared to 80 mM NaCl conditions. However, when compared to unfixed chromatin in these conditions, click-fixation did partially reduce chromatin de-compaction (Figures 5.15, 5.27) and suggests that a click-based approach could fix chromatin fibre structure. The lack of complete fixation could be due to not producing enough DNA click crosslinks within a 1-hour reaction. While a longer reaction time could increase the number of crosslinks, this might also induce a bias similar to the long fixation times in formaldehyde (Baranello et al., 2016). Moreover, analysis in 1 mM NaCl treatment may be too excessive an alteration compared to physiological conditions. As DNA crosslinks would not prevent proteins dissociating from the chromatin fibre, the loss of linker histones or the production of heminucleosomes in 1 mM NaCl could be responsible for partial chromatin de-compaction.
Chapter 6: Discussion

6.1 Heterochromatin regulators

The cytological restriction of centromeric DNA during metaphase identifies the centromere as having a distinct chromatin structure. In some organisms centromeres remain cytologically compact throughout the cell cycle leading to their label as constitutively compact (or heterochromatic) DNA regions. This provides a good distinction from which to investigate chromatin structure and function, leading initial studies to focus on effectors of heterochromatin formation and maintenance (Muller, H, 1930). Using the position-effect variegation (PEV), determined by an eye pigment gene translocation to centromeric regions, gave a phenotypic output to identifying factors regulating heterochromatin formation and regulation.

Since the development of sequencing based ‘omics’ approaches, our understanding of euchromatin organisation has advanced significantly. However the repetitive nature of centromeric DNA sequences has prevented chromatin conformation and modification distributions to be resolved at these regions. Therefore identifying factors and regulators of heterochromatin structure remains an important question within the chromatin field.
Model systems, such as artificial chromosomes, have often been used to investigate heterochromatin structure (Grimes, Rhoades and Willard, 2002; Ohzeki et al., 2002). However, measuring the stability of artificial chromosomes to identify centromeric components is difficult, and it has limitations for identifying heterochromatin components. This approach also lacks scalability, and is limited to essential components of faithful chromosome segregation rather than heterochromatin structure directly. The artificial heterochromatin system developed by the Larionov lab enables large scale screening of heterochromatin factors, while its artificial nature should limit cell type specific regulation. This artificial system uses a centromeric sequence from yeast chromosome 6 and a 10 kb array of human alpha satellite, and mimics the epigenetic profiles of constitutive and facultative heterochromatin resulting in transcriptional silencing (Figure 3.9) (Ebersole et al., 2005). A GFP reporter can then be used to provide a fast and efficient output of gene expression.

5’azacytidine and trichostatin A, well characterised inhibitors of DNA methyltransferases and HDACs respectively, triggered GFP expression. This demonstrates that inhibition of canonical heterochromatin components results in GFP expression within this system and helps to validate it as a screening tool (Figure 3.2). Additionally many specific HDAC inhibitors gave GFP expression with this screen (Table 3.1).

The identification of kinase signalling pathways in modulating heterochromatin is not surprising given that cells utilise kinase signalling to adapt to their environments and alter transcription. As transcriptional changes require chromatin remodelling it is also not surprising that some kinase pathways are involved in heterochromatin maintenance. Indeed
aurora kinase has well characterised roles in regulating cohesin loading and proper chromatin compaction (Nishiyama et al., 2010; Tedeschi et al., 2013).

The surprising result from the work presented here was the identification of BET proteins in maintaining heterochromatic gene repression. Both pan-BET inhibitor bromosporine and selective inhibitor JQ1 de-repressed GFP expression and caused G1 cell cycle arrest demonstrating the effect is not compound specific (Figure 3.4, 3.5). BET proteins canonically function as transcriptional activators (Chapuy, McKeown and Lin, 2013) where they bind to acetylated histones and are therefore often associated with euchromatin (Najafova et al., 2017). Additionally Wang et al., (2012) demonstrated that full length Brd4 protein was required to maintain chromatin structure and prevent abnormal heterochromatinisation.

However some recent studies suggest that histone acetylation and BET proteins can also be associated with pericentric heterochromatin maintenance (Grézy et al., 2016). This study demonstrated that when H3K9me3/Suv39h/HP1 heterochromatin association is disrupted, that TIP60 acetylation recruits BET proteins to maintain heterochromatin compaction.

Further studies have demonstrated specific recruitment of BET proteins to heterochromatin due to multiple stress responses (Floyd et al., 2013). This study demonstrated that Brd4 can recruit condensin II preventing DNA damage response pathways, while Col et al., (2017) demonstrated BET proteins were essential for pericentric transcription after heat shock. Together these studies support the idea that BET proteins can function at heterochromatin in certain situations.
The synthetic GFP cassette in the MEL cells is marked by H3K9me3 enrichment suggesting Suv39h/HP1 mediates its heterochromatinisation. This therefore implicates BET proteins function beyond abnormal heterochromatin organisation and stress responses.

When global histone modifications were analysed by protein arrays, H3K4 methylation was elevated (Figure 3.10). This could be due to inhibition of BET proteins binding to acetylated lysines, which exposes histone acetylation to increased de-acetylation. H3K4 acetylation is associated with pericentric heterochromatin in yeast, and has an attributed function in recruiting the HP1 homologue Swi6 (Xhemalce and Kouzarides, 2010). If BET proteins bind to H3K4ac at pericentric regions this could provide a potential mechanism of action for heterochromatin maintenance.

Additionally BET inhibitors resulted in significant decreases in MAPK signalling, and compensatory up regulation of PI3K/Akt and JNK signalling (Table 3.2) (Chen and Sytkowski, 2001; Mirzoeva et al., 2009). BET proteins have a key function in regulating c-Myc transcription, which is downstream of MAPK signalling. In turn, c-Myc is stabilised by MEK1 phosphorylation, but acts as a negative feedback loop for MAPK signalling by supressing ERBB-kinases (Tsai et al., 2012; C. Sun et al., 2014). This provides a mechanism for BET protein mediated activation of MAPK signalling pathways. As aberrant MAPK signalling is associated with driving EMT-like transitions in cancer, the role of BET inhibitors in MEK inhibition resistance was investigated.
6.2 The relationship between BET proteins and EMT driven drug resistance

Epithelial mesenchymal transitions (EMT) is a natural developmental process first observed in the heart (Markwald, Fitzharris and Smith, 1975). The process was initially described as the invasion of the cardiac matrix between E8.5 and E12, when cells morphologically change and aggregate into fibroblastic cushions. However this pathway is often hijacked by cancer cells to promote invasion and tumour metastasis (Yang et al., 2004; S. H. Wang et al., 2017).

The EMT phenotype is the loss of cellular adhesion and membrane polarisation via reduced levels of E-cadherin and claudin proteins. Additionally N-cadherin, fibronectin, and extracellular matrix proteases are up regulated. Snail or Twist regulates this phenotypic switch and is strongly associated with metastasis (Rosivatz et al., 2002; Yang et al., 2004). Cells that have undergone an EMT-like transition often require the hyper activation of the Ras/Raf/MEK/ERK signalling pathway (Oft et al., 1996; Seton-Rogers et al., 2004; Xie et al., 2004). In addition, compensatory kinase pathways (Pi3K and Src) that are known to have crossover with the Ras/Raf pathway have also been implicated as drivers of EMT and metastasis (Bakin et al., 2000; Fang et al., 2017).
EMT-like transitions occur with a high frequency in cutaneous melanomas, leading to aggressive metastasis and poor patient prognosis (Dissanayake et al., 2007; Fenouille et al., 2012). As 45% of patients present with BRAF mutations and 20% present with NRAS mutations, MAPK inhibition has been explored as a therapeutic avenue (Hocker and Tsao, 2007; Flaherty et al., 2012). Unfortunately, melanomas rapidly develop resistance and metastasise by hyper activating the MAPK pathway and only 20% present with new genetic abnormalities (Shi et al., 2012; Long et al., 2014). This suggests that acquired drug resistance occurs on a genome regulation level, rather than by aberrant genetic instability. Additional studies indicate that EMT-like status was completely independent of MAPK mutational status and is reversible (Hoek et al., 2006; Fallahi-Sichani et al., 2017). This further suggests that MAPK acquired drug resistance linked EMT-like transitions may be epigenetically regulated.

BET inhibition and loss of artificial heterochromatin silencing in the α-MEL system (Table 3.2) resulted in hyper phosphorylation of MAPK signalling, in addition to up regulation of EMT biomarkers including JNK signalling, FAK1, and STAT signalling. This suggested that BET proteins might function during EMT.

An HCT116 cell line, developed by John Dawson, has n-Ras driven MAPK signalling that when exposed to MEK inhibitor trametinib, developed resistance with a mesenchymal phenotype (Figure 4.1). This provided a useful model to utilise epigenetic inhibitors to determine EMT-like pathway epigenetic components in relation to patient phenotype.
As one might expect, crossover kinase inhibitors (PLK/Akt) were more sensitive in resistant cells with hyper phosphorylation of MAPK signalling (Figure 4.3). Additionally identifying polycomb as functioning in acquired drug resistance is supported by recent evidence implicating PRC2 as a regulator of EMT and poor patient prognosis (Jene-Sanz et al., 2013; Tiwari et al., 2013; Cardenas et al., 2016).

BET inhibition sensitivity increased further in mesenchymal-like cells and correlates with the kinase signalling response identified in α-MEL cells (Figure 4.8). The use of multiple BET inhibitors enabled off-target effects to be ruled out, and were used to investigate the role of specific BET proteins based on inhibitor selectivity. This demonstrated that selectivity toward Brd2 correlated with cell survival (Figure 4.7). This specificity towards Brd2 has recently been supported by studies suggesting that Brd2 and Brd4 function as opposite drivers of EMT and MET respectively. Andrieu and Denis, (2018) demonstrated that EMT-like transitions in triple negative breast cancer are induced by an up regulation of Brd2 expression, a process repressed by Brd4. This is also supported in non-disease cellular environments. Brd4 to Brd2 occupancy switching at enhancers, including Nodal, results in mesodermal differentiation of mES cells (Fernandez-Alonso et al., 2017). This would explain the antagonistic effect observed in low concentrations in combination with trametinib (Figure 4.5).

Furthermore, c-Myc, the downstream effector protein for Ras/Raf/MEK/ERK signalling is up regulated by acquired MAPK inhibitor resistance (Singleton et al., 2017; Zawistowski et al., 2017). BET proteins are known to have an essential function in regulating c-Myc expression, presenting another dependency of EMT-like acquired drug resistance on Brd2/4 (Lovén et al.,
There is growing evidence that cells having gone through an EMT-like transition, can overcome the loss of EMT drivers such as Notch/TGF-β signalling via enhancer BET protein occupancy (Yashiro-Ohtani et al., 2014). Together this evidence supports the data presented here that BET proteins, and specifically Brd2, have a key regulatory role in EMT-like transitions and resistance to MEK inhibition.

When BET protein levels were observed in endothelial like (HCT116) and mesenchymal like (TRAMR) cells, Brd2 transcription and nuclear protein levels were elevated in the trametinib resistant mesenchymal like cells. Additionally Brd2 protein and transcript levels were both reduced upon BET inhibition in TRAMR cells indicative of a specific function on TRAMR cells (Figures 4.12, 4.13). Intriguingly this correlated with HP1α and HP1γ protein levels. Given HP1 proteins key role in chromatin compaction, this suggests that BET proteins may function to modulate chromatin organisational changes in mesenchymal like cells. Comparison of multiple endothelial and mesenchymal cell types demonstrated that BET protein occupancy at HP1 promoters only occurred in mesenchymal cells and that bromodomain inhibition reduced Brd2 occupancy (Figures 4.15, 4.16) (W. Liu et al., 2013; Asangani et al., 2014; Knoechel et al., 2014).

HP1 proteins and large scale conformation changes in chromatin organisation are associated with EMT (McDonald et al., 2011). Maybe, this is not surprising given the stark and relatively stable phenotypes of endothelial and mesenchymal cells. TGF-β induced EMT causes initial repression of pericentric transcription mediated by Snail1 recruitment and HP1α release (Millanes-Romero et al., 2013). This demonstrates the potential for MAPK signalling to regulate the large scale chromatin reorganisation during EMT.
Furthermore BRCA1 functions to suppress EMT with mutations associated with poor patient prognosis (Bai et al., 2014). Loss of BRCA1 elevates major satellite transcription in mice while it also associates with HP1γ to form facultative heterochromatin mediated silencing of interspersed retrotransposons (Zhu et al., 2011; Filipponi et al., 2013).

To determine BET protein distribution immunofluorescence across the nucleus was used in endothelial and mesenchymal like cells (Figure 4.10). While no discernible distribution pattern was observed for Brd2 or Brd3, JQ1 treatment resulted in the formation of Brd4 foci that were associated with the nuclear periphery in mesenchymal like cells. This suggests a direct function for BET proteins at heterochromatin, supported by the recent study demonstrating TIP60 mediated BET protein recruitment to heterochromatin (Grézy et al., 2016). Together these data support the current opinion that BET proteins have a function in EMT-like transitions, while also suggesting that this function may be important for heterochromatin regulation and nuclear organisation.
6.3 H3K9me3 and chromatin compaction

Our understanding of heterochromatin structure and regulation currently remains limited. A key model system for investigation of HP1 proteins role at pericentric heterochromatin, are mouse embryonic stem cells and their Suv39h1 and Suv39h2 double null sister cells (Peters et al., 2001).

Unlike human cells mouse constitutive heterochromatin is readily visible by microscopy and DAPI staining (Figures 1.4, 5.13). Despite the classical studies identifying Suv39h methyltransferases and HP1 proteins as suppressors of variegation, the cytologically distinct aggregates of constitutive heterochromatin are not lost upon Suv39h/H3K9me3/HP1 loss from these domains (Figures 5.3, 5.11). These domains in Suv39h knockouts also lack DNA methylation, H4K20me3, and increased histone acetylation (Peters et al., 2001). Given these large-scale changes to the heterochromatin epigenetic profile, the maintained presence of chromocenters, questions the relevance of histone modifications and their binding partners having a causal role in chromatin structure regulation. Indeed studies have directly demonstrated that loss of DNA methylation from heterochromatin had no effect on chromatin structure (Gilbert, Thomson, Boyle, Allan, Ramsahoye and Wendy A. Bickmore, 2007).

To determine if H3K9me3 and HP1 proteins had a functional structural role at heterochromatin, the chromatin compaction state of wild type (WT41) and Suv39h1 and Suv39h2 double null (DN57)) mouse ES cells was analysed (Figures 5.5, 5.6). It was demonstrated that there was a resolvable loss in chromatin compaction in DN57 cells. This chromatin de-compaction was not
however limited to pericentric heterochromatin and suggested a global effect on chromatin compaction. Although H3K9me3 and HP1 is also found at facultative heterochromatin and interspersed retrotransposons, de-compaction of these additional regions would still not account for the global change in chromatin compaction. This suggests that the effects of correct heterochromatin formation and H3K9me3/HP1 association extend beyond heterochromatin, effecting euchromatic regions. This affect was abrogated by the reestablishment of H3K9me3 by exogenous human Suv39h1 expression (Figure 5.12). This is the first evidence that alterations in histone modifications can affect chromatin structure.

There is a growing body of evidence for an RNA component in chromatin compaction (Maison, Bailly, Peters, J. P. Quivy, et al., 2002; Arnoult, Van Beneden and Decottignies, 2012). Both Suv39h and HP1 protein families have RNA binding domains, but the exact function of RNA is hotly debated (Maison et al., 2011; Johnson et al., 2017; Shirai et al., 2017; Velazquez Camacho et al., 2017). Chromatin associated RNA in DN57 cells was analysed and preliminary results indicated that H3K9me3 loss correlated with a global elevation in chromatin associated RNA (Figures 5.13, 5.14).

As previously stated, transitions to a mesenchymal-like phenotype require elevated levels of pericentric transcription and chromatin reorganisation (Zhu et al., 2011). The expression of heterochromatic repetitive elements is inversely correlated with H3K9me3 and HP1 association in a mesenchymal setting (Filipponi et al., 2013). This suggests that while RNA functions in heterochromatin formation, transcription of heterochromatic elements requires its absence (Casanova et al., 2013; Velazquez Camacho et al., 2017). Loss of H3K9me3 from constitutive heterochromatin is therefore likely to
increase satellite transcription. These repeat transcripts are non-coding and have been observed up to 8 kb in length in mouse cells (Quivy et al., 2004).

Chromatin associated RNA has long been identified as a major chromatin component in vivo (Holmes et al., 1972; Berezney and Coffey, 1974). Recent work by Nozawa et al., (2017) demonstrated that chromatin associated RNA interacts with scaffold attachment factor A (SAF-A) to de-compact chromatin. This mechanism, coupled with increased satellite transcripts in the absence of H3K9me3 presents a potential mechanism for global chromatin de-compaction beyond the Suv39h wild type mediated domains of H3K9me3.
6.4 Perspectives

Regulation of chromatin structure is a complex multifaceted system utilising many biochemical and spatial components. These include; the transcriptional status, radial nuclear distribution, histone modifications, RNA containing nuclear scaffolds, cohesin and condensin mediated TAD formation, and most recently phase separation based on hydrophillicity (Wiblin et al., 2005; Larson et al., 2017; Nozawa et al., 2017; Rao et al., 2017). The interplay between these components has yet to reveal a causal driver of large-scale chromatin regulation.

However one can think of chromatin fibre structure regulation as a similar process to protein folding. Multiple orders of protein folding are required, and the local environment and physical forces must be tightly regulated to ensure a correct structure. In this manner, recent work understanding chromatin organisation into phase separated states with different hydrophilic forces suggests that chromatin regulators can alter the chromatin local environment to affect its structure (Larson et al., 2017). This is consistent with protein folding where hydrophilicity is the greatest force effecting folding across the sequence (Dyson, Wright and Scheraga, 2006) and in proteins this results in the aggregation of hydrophobic residues to form secondary and tertiary structures (Camilloni et al., 2016).

As such it is important to re-evaluate chromatin structure regulatory processes within a similar context. Histone acetylation or phosphorylation states for instance alters histone hydrophilicity (Lindner et al., 1996). Additionally the disordered hydrophobic C-terminal domain of linker
histone H1 is required for chromatin compaction (Allan et al., 1986). This suggests that H1 proteins alter the hydphilicity of the chromatin supramolecule to facilitate compaction. In this same way, H3K9me3 recruited HP1 proteins have an essential unstructured hinge region, that interacts with RNA (Meehan, Kao and Pennings, 2003). The oligomierisation of HP1 into large arrays in the supramolecule are therefore likely to change the fibres hydphilicity facilitating conformational changes or sequestration into liquid droplets (Strom et al., 2017).

The large RNA component of chromatin, suggests that it would also have a significant effect on the chromatin fibre supramolecules hydphilicity. The base pairs of nucleic acids are naturally hydphobic while the polar phosphate backbone is hydphilic. In physiological conditions DNA generally forms B-form helices with charges distributed throughout. RNA, in contrast, forms A-form helices where the base pairs are more exposed. RNA secondary structures can therefore dramatically alter their hydphilicity profile dependent on conformation (Suga et al., 2012). This switching between hydphobic and hydphilic states could therefore be regulatory by sequestering chromatin into liquid phase states. Altered RNA conformation by methylation or protein binding may also affect the physical properties of the underlying complex or molecule, providing a mechanism of action for RNA mediated tethering or aggregation.

While the studies presented here demonstrate that histone modifications can directly alter chromatin fibre compaction, it is possibly indirect via altering non-coding RNA transcription. Pericentric non-coding RNA transcription has been presented as a driver of chromatin reorganisation in EMT/MET-like processes (Millanes-Romero et al., 2013). This supports observations herein of
Brd4 foci formation at the nuclear periphery once global Brd2 levels were reduced.

This work suggests that a Brd2/Brd4 switch functions in altering chromatin organisation and is supported by recent publications (Cheung et al., 2017; Fernandez - Alonso et al., 2017). It also suggests that Brd2 is a key regulator of EMT-like acquired drug resistance, but understanding mechanistically how Brd2 modulates this process would be the next step. These studies investigating the fundamental components of heterochromatin provide further evidence that RNA can modulate chromatin structure; the mechanism behind such regulation remains the elusive goal of the heterochromatin field.
Appendices

Appendix 1. α-MEL eGFP reactivation compound screen. eGFP expression after α-MEL cells were screened with each compound in turn for 48 hours, and fluorescence determined by flow cytometry. Table of initially screened compounds (n = 1) GFP fluorescence after treatment for 48 hours at 1 µM (unless stated otherwise).

<table>
<thead>
<tr>
<th>Compound</th>
<th>Δ Mean GFP</th>
<th>Compound</th>
<th>Δ Mean GFP</th>
</tr>
</thead>
<tbody>
<tr>
<td>(-)-Neplanocin A</td>
<td>0.81</td>
<td>Au7</td>
<td>-0.52</td>
</tr>
<tr>
<td>(5Z)-7-Oxozeaenol (BIX)</td>
<td>0.53</td>
<td>Au8</td>
<td>-0.65</td>
</tr>
<tr>
<td>0572-0022</td>
<td>-1.09</td>
<td>Au9</td>
<td>-0.54</td>
</tr>
<tr>
<td>2-PCPA</td>
<td>0.58</td>
<td>Aurora</td>
<td>4.10</td>
</tr>
<tr>
<td>2,4 DPD</td>
<td>0.29</td>
<td>BAY 11-7082</td>
<td>1.07</td>
</tr>
<tr>
<td>2,4-Pyridinedicarboxylic Acid</td>
<td>0.34</td>
<td>Benzo(a)pyrene</td>
<td>16.82</td>
</tr>
<tr>
<td>3-amino Benzamidine</td>
<td>0.33</td>
<td>Bromosporin</td>
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</tr>
<tr>
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<td>Bromosporin (0.2uM)</td>
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<td>Bromosporin (0.5uM)</td>
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<td>Bromosporin (1.5uM)</td>
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<td>-0.58</td>
</tr>
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<td>-0.60</td>
</tr>
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<td>-0.59</td>
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Appendix 2. Significant kinase changes to BET inhibition in α-MEL cells. α-MEL cells were treated with I-BET151 or JQ1+/− 1 µM for 24 hours. Protein lysates were spotted onto arrays and changes in kinase signaling after JQ1 treatment when compared to DMSO controls. JQ1 and IBET151 treated α-MEL cells were treated as replicates and binned together before compared to DMSO controls. Bonferoni two-tail T-test analysis performed (*, p < 0.05) (**, p < 0.01).

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<td>CrkL P Tyr207</td>
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<td>p44/42 MAPK (ERK1/2) P Thr202/Thr185,Tyr204/Tyr187</td>
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<td>p-value (Bonferroni)</td>
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<td>MNK1 (MKNK) P Thr197, Thr202</td>
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<td>Src (family) P Tyr416</td>
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<td>mTOR P Ser2481</td>
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<td>Smad3 P Ser423, Ser425</td>
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<td>Stat1 P Ser727</td>
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<td>Stat5</td>
<td>-12.56719</td>
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<td>beta-Tubulin</td>
<td>60.8556</td>
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<td>BRCA1</td>
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<td>PKC-gamma P Thr514</td>
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<td>Stat6 P Tyr641</td>
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<td>Zap70</td>
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<tr>
<td>PDGFR P Tyr751</td>
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<td>3.82085E-9 **</td>
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<tr>
<td>SAPK/JNK</td>
<td>30.08464</td>
<td>0.00565 *</td>
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Appendix 3. Kinase signaling changes in response to trametinib. Cells were treated with DMSO or 300 nM Trametinib for 24 hours before protein lysates were prepared and spotted onto arrays. List of all changes to kinase antibody fluorescence between either: (1) basal states of HCT116 and its trametinib resistant sister cells TRAMR, (2) HCT116 cells trametinib 300nM 24 hour treatment to DMSO controls, or (3) TRAMR cells trametinib 300 nM 24 hour treatment to DMSO controls.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Basal Δ TRAMR p-value</th>
<th>HCT116 Δ Trametinib p-value</th>
<th>TRAMR Δ Trametinib p-value</th>
</tr>
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<tbody>
<tr>
<td>PKM2 XP(R)</td>
<td>1.09 0.874</td>
<td>1.51 0.468</td>
<td>0.98 0.965</td>
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<td>SQSTM1</td>
<td>0.98 0.969</td>
<td>1.32 0.643</td>
<td>1.25 0.594</td>
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<td>PLC-gamma1</td>
<td>1.16 0.782</td>
<td>0.85 0.832</td>
<td>0.93 0.874</td>
</tr>
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<td>Puma</td>
<td>1.17 0.777</td>
<td>0.97 0.961</td>
<td>0.87 0.784</td>
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<tr>
<td>VEGFR P Tyr951</td>
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<td>1.33 0.632</td>
<td>1.23 0.632</td>
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<tr>
<td>Plk1 P</td>
<td>1.37 0.530</td>
<td>0.37 0.367</td>
<td>0.73 0.572</td>
</tr>
<tr>
<td>Calpastatin</td>
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<td>1.48 0.492</td>
<td>1.01 0.977</td>
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<td>ErbB-2/Her2/EGFR</td>
<td>0.63 0.527</td>
<td>1.72 0.305</td>
<td>1.53 0.258</td>
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<td>FRα1 (R20)</td>
<td>0.93 0.904</td>
<td>1.06 0.937</td>
<td>1.11 0.816</td>
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<td>cdc2</td>
<td>0.97 0.958</td>
<td>1.62 0.378</td>
<td>1.26 0.583</td>
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<td>1.25 0.666</td>
<td>0.86 0.844</td>
<td>0.76 0.614</td>
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<td>Fox01 P</td>
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<td>0.88 0.866</td>
<td>0.80 0.678</td>
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<td>HDAC4/5/6/7 P</td>
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<td>0.82 0.797</td>
<td>0.77 0.630</td>
</tr>
<tr>
<td>Ezrin/ERM P</td>
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<td>1.62 0.372</td>
<td>1.19 0.684</td>
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<tr>
<td>ErbB-2/Her2/EGFR P</td>
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<td>3.79 &lt;0.001</td>
<td>2.09 0.021</td>
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<tr>
<td>Tyr1248/Tyr1173</td>
<td>0.82 0.761</td>
<td>1.79 0.261</td>
<td>1.22 0.648</td>
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<tr>
<td>IGF-1R beta P</td>
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<td>0.96 0.954</td>
<td>0.95 0.922</td>
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<td>1.04 0.931</td>
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<td>0.58 0.550</td>
<td>0.55 0.345</td>
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<td>GSK-3-alpha/beta P</td>
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<td>1.88 0.210</td>
<td>1.35 0.457</td>
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<td>0.91 0.900</td>
<td>0.93 0.889</td>
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<td>Stat6</td>
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<td>1.56 0.237</td>
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<td>0.76 0.611</td>
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<td>XIAP</td>
<td>0.86 0.817</td>
<td>0.68 0.642</td>
<td>0.84 0.729</td>
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<td>CamKII P Thr286</td>
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<td>2.19 0.089</td>
<td>1.64 0.176</td>
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<td>1.12 0.864</td>
<td>0.96 0.937</td>
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<td>1.31 0.653</td>
<td>0.96 0.934</td>
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<td>1.69 0.146</td>
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<td>1.03 0.968</td>
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<tr>
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<td>1.36 0.441</td>
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<td>HCT116 $\Delta$ Trametinib $p$-value</td>
<td>TRAMR $\Delta$ Trametinib $p$-value</td>
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<td>---------------------------------</td>
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<td>S6 Ribosomal protein $P$</td>
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<td>Ser235,Ser236</td>
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<td>0.06 0.178</td>
<td>0.30 0.141</td>
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<td>beta-actin</td>
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<td>c-Jun $P$ Ser73</td>
<td>1.34 0.558</td>
<td>1.67 0.340</td>
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<td>Tsc-2 (Tuberin)</td>
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<td>0.56 0.531</td>
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<td>1.08 0.890</td>
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<td>1.06 0.891</td>
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<td>beta-Tubulin</td>
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<td>0.76 0.729</td>
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<td>p90 S6 kinase (Rsk1-3) $P$</td>
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<td>Ki-67 (Annexin II, p36)</td>
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<td>HCT116 $\Delta$ p-value Trametinib</td>
<td>TRAMR $\Delta$ p-value Trametinib</td>
</tr>
<tr>
<td>-------------------------------</td>
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<td>1.01</td>
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<tr>
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<th>HCT116 $\Delta$ Trametinib $p$-value</th>
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